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## Conformational characterisation of valinomycin complexation with barium salts—A nuclear magnetic resonance spectroscopic study

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**Abstract.** Conformations of valinomycin and its complexes with perchlorate and thiocyanate salts of barium, in a medium polar solvent acetonitrile, were studied using nuclear magnetic resonance spectroscopic techniques. Valinomycin was shown to have a bracelet conformation in acetonitrile. With the doubly charged barium ion, the molecule, at lower concentrations, predominantly formed a 1 : 1 complex. At higher concentrations, however, apart from the 1 : 1, peptide as well as ion sandwich complexes were formed in addition to a 'final complex'. Unlike the standard 1:1 potassium complex, where the ion was centrally located in a bracelet conformation, the 1 : 1 barium complex contained the barium ion at the periphery. The 'final complex' appeared to be an open conformation with no internal hydrogen bonds and has two bound barium ions. This complex was probably made of average of many closely related conformations that were exchanging very fast (on nuclear magnetic resonance time scale) among them. The conformation of the 'final complex' resembled the conformation obtained in the solid state. Unlike the perchlorate anion, the thiocyanate anion seemed to have a definite role in stabilising the various complexes. While the conformation of the 1 : 1 complex indicated a mechanism of ion capture at the membrane interface, the sandwich complexes might explain the transport process by a relay mechanism.

**Keywords.** NMR study; conformation; ionophores; valinomycin-barium complex; trans-membrane ion-transport.

### Introduction

Valinomycin is a cyclic dodecadepsipeptide, which has been well studied as a model system for understanding biological transport across membranes in higher organisms (Ovchinnikov *et al.*, 1974; Stark, 1978; Ovchinnikov, 1979; Lauger, 1980; Lauger, *et al.*, 1981). Two most important characteristics of the molecule that have come out of these studies are (i) its selective transport of  $K^+$  over  $Na^+$  (by a factor of over  $10^4$ ), both in natural (Pressman, 1965) as well as synthetic (Mueller and Rudin, 1967) membranes, which explains its antibiotic activity and (ii) its solvent dependent conformations (Patel and Tonelli, 1973; Ovchinnikov, *et al.*, 1974) that help provide explanations for the mechanism of transport. While it is true that some of the conformations of valinomycin have been characterised to a great extent (Duax *et al.*, 1972; Patel and Tonelli, 1973; Ovchinnikov and Ivanov, 1974; Ovchinnikov *et al.*, 1974; Karle, 1975; Neupert-Laves and Dobler, 1975; Smith *et al.*, 1975; Bystrov *et al.*, 1977), all the conformational

possibilities for the molecule have not been explored sufficiently. Providing an exact mechanism of transport by the molecule depends on knowing the various conformational rearrangements the molecule has to undergo within the membrane, and this can possibly be achieved by studying the conformation of the molecule under a variety of solution and salt conditions. To this end, we have characterised the barium complexes of valinomycin in acetonitrile solvent.  $\text{Ba}^{2+}$  (1.38 Å) has almost the same ionic radius as  $\text{K}^+$  (1.33 Å) but has a double charge and therefore the effect of increased charge on the complex conformation of the molecule can be well studied. Some of the results obtained using circular dichroism (CD), X-ray and nuclear magnetic resonance (NMR) techniques have already been reported (Devarajan *et al.*, 1980; Devarajan and Easwaran, 1981; Devarajan *et al.*, 1983). In this paper, we report a detailed NMR study of barium complexation with valinomycin.

## Materials and methods

Valinomycin was obtained from Sigma Chemical Company, St. Louis, Missouri, USA and was used as such. Only perchlorate and thiocyanate salts were used for complexation studies as they showed low cation-anion interactions. The perchlorate salt was prepared by neutralising barium hydroxide with perchloric acid and then drying. The thiocyanate salt was a gift from Shemyakin Institute of Biorganic Chemistry, Moscow, USSR. The salts were dried in vacuum over phosphorous pentoxide for several days before use.

All the deuterated solvents were purchased from Stohler Isotope Company, Massachusetts, USA and were used as such. The free radical 2,2,6,6 tetramethyl piperiden-1-oxyl was a gift from Prof. K. D. Kopple. The NMR spectra were recorded using Bruker WH 270 FT-NMR spectrometer of the Sophisticated Instrumentation Facility, Indian Institute of Science, Bangalore, under controlled FT mode, at 270 MHz for  $^1\text{H}$  and 67.89 MHz for  $^{13}\text{C}$ . Five mm and 10 mm diameter NMR tubes from Wilmad Glass Company were used for  $^1\text{H}$  and  $^{13}\text{C}$  respectively. The  $^{13}\text{C}$  NMR spectra were recorded with broad band decoupling of the coupled protons. For all experiments, deuterium signals from solvents were used for locking the magnetic field of the spectrometer. The chemical shifts were measured in parts per million (ppm) with respect to internally added tetramethyl silane (TMS). The experiments were performed in  $\text{CD}_3\text{CN}$  unless otherwise stated. The stoichiometrics mentioned in this paper correspond to valinomycin: metal salt in that order only.

## Results and discussion

### Proton NMR studies

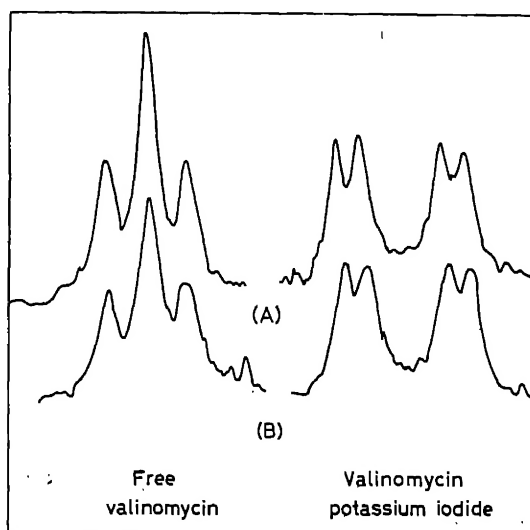
*Conformation of valinomycin in acetonitrile:* The conformation of valinomycin was determined in deuterated acetonitrile ( $\text{CD}_3\text{CN}$ ) a solvent used through out this study as valinomycin assumes solvent dependent conformations.

Table 1 lists the  $\text{C}^\alpha$  proton chemical shifts and coupling constants of valinomycin in various conformations. Apart from the similarity of  $\text{C}^\alpha$  proton chemical shifts, the

**Table 1.** A comparison of chemical shifts and coupling constants for valinomycin in various states.

State	$^{13}\text{C}$ -H Chemical shifts (ppm)				Coupling constants $^3J_{\text{HNC}^{\alpha}\text{H}}$ (Hz)		References
	L-Lac	D-HyIv	D-Val	L-Val	L-Val	D-Val	
A	5.31	5.03	4.03	3.91	7.0	8.5	Bystrov <i>et al.</i> , 1977
B	5.09	4.82	4.26	4.36	7.5	10.1	Bystrov <i>et al.</i> , 1977
$\text{CD}_3\text{CN}$	5.25	5.00	4.11	4.14	7.6	7.6	Present studies

coupling constants are also very close for valinomycin in  $\text{CD}_3\text{CN}$  and A conformation. Also, a free radical (2,2,6,6, tetramethyl piperiden-1-oxyl) which is capable of broadening any exposed amide proton (Kopple *et al.*, 1978) on addition to valinomycin and its  $\text{K}^+$  complex in  $\text{CD}_3\text{CN}$  under identical valinomycin concentration conditions, does not cause any broadening in both the systems (figure 1). This observation indicated that all the valinomycin amide protons were hydrogen bonded in  $\text{CD}_3\text{CN}$  just as in its  $\text{K}^+$  complex. Figure 2 shows the effect of changing the solvent from nonpolar  $\text{CDCl}_3$  (chloroform) to  $\text{CD}_3\text{CN}$ , on the carbonyl carbons chemical shifts. The amide carbonyl carbons [L-lactic (Lac) and D-hydroxyisovaleric acid (HyIv)] that are involved in hydrogen bonding shift only slightly, indicating that the hydrogen bonding scheme is same in both the solvents. The shifts in the ester carbonyl carbons

**Figure 1.** Effect of free radical addition to valinomycin and its  $\text{K}^+$  complex both in  $\text{CD}_3\text{CN}$ . Valinomycin concentration is 8.9 mM. A. Before addition. B. After addition.

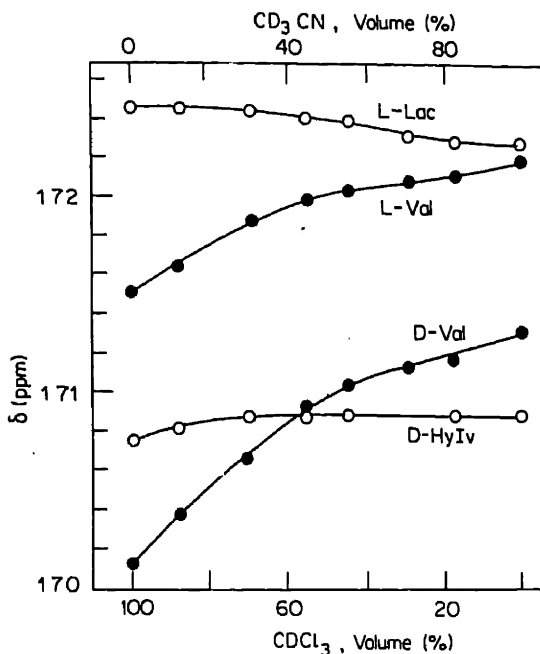


Figure 2. Solvent titration for the assignment of carbonyl signals of free valinomycin in  $\text{CD}_3\text{CN}$ ; X axis: volume per cent of  $\text{CDCl}_3$  or  $\text{CD}_3\text{CN}$ ; Y axis: (ppm) with reference to TMS.

[L- and D-valine (Val)] seen in figure 2, indicated a changed orientation for these groups. Thus valinomycin assumes A conformation in  $\text{CD}_3\text{CN}$  ( $\text{CH}_3\text{CN}$ ) with all its hydrogen bonds intact.

*Studies on valinomycin-barium complexes:* It has been known that chemical shift positions of the  $\text{C}^\alpha$  protons are sensitive to the conformation of the molecule, in particular to the change in orientation of the adjacent carbonyls (Grell and Funck, 1973a). The carbonyls can change their orientation in metal complexation studies to, either ligand effectively with the cation, or accommodate the resultant conformational changes by adjusting the hydrogen bond scheme. Therefore, conformational characterisation of the various complexes can be achieved, by following the chemical shift changes at the various  $\text{C}^\alpha$  protons. The shifts in the amide protons give an idea about hydrogen-bond scheme.

#### *Studies on valinomycin-barium perchlorate complexes*

*At lower valinomycin concentrations:* Figure 3 shows the chemical shift changes for the NH and  $\text{C}^\alpha\text{H}$  signals of valinomycin (0.37 mM) with barium perchlorate addition. A plateau at 1:1 indicated stabilisation of the 1:1 complex. Three points emerged from a comparison of these chemical shifts with those corresponding to  $\text{K}^+$  addition (Ovchinnikov and Ivanov, 1974). (1) The amide protons (L- and D-Val NH's) showed a



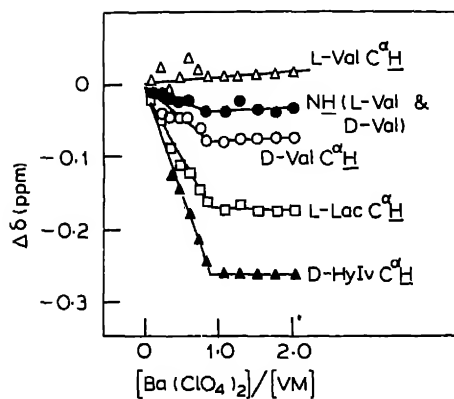


Figure 3. Barium perchlorate addition to valinomycin at 0.37 mM concentration. The stabilisation of the signals at 1:1 is clearly seen.

large low-field shift with the  $K^+$  complex formation, but have only a small high-field shift with the  $Ba^{2+}$  complex formation. (2) While the L-Val  $C^\alpha H$  showed a considerable high-field shift (0.28 ppm) with the  $K^+$  complex formation, it almost did not produce shift with the  $Ba^{2+}$  complex formation. (3) The shift in D-Val, L-Lac and D-HyIv  $C^\alpha H$ 's was in the same direction in both the complexes. However, the magnitude of shifts for the barium complex was smaller.

The larger low-field shift of the amide protons in the  $K^+$  complex could be attributed to the electron withdrawing effect of the nearby  $K^+$  ion. The doubly charged barium ion (which could have caused a still larger low-field shift, if it were to occupy the  $K^+$  position) could be only at the periphery of the bracelet. The absence of a change in the chemical shift position of the L-Val  $C^\alpha H$  indicated that the binding was not from the L-Val side definitely, and could be from the D-Val side.

The small high-field shift of the amide protons indicated some weakening of hydrogen bonds. This result suggested that the amide (L-Lac and D-HyIv) were turning away from an hydrogen bonded position, to co-ordinate with the barium ion. The large high-field shifts in L-Lac and D-HyIv  $C^\alpha H$ 's seemed to confirm such a possibility. The shift in the D-Val  $C^\alpha H$  though high-field as in the  $K^+$  complex, was smaller. This study indicated that this ester carbonyl, though turned inward as in the  $K^+$  complex, did not have to change its orientation markedly.

All these inferences indicated a 1:1 barium complex in which the barium ion was positioned at the periphery of the bracelet, on the D-Val side, complexing with D-Val, L-Lac and D-HyIv carbonyl oxygens (Devarajan and Easwaran, 1981). The ion possibly still retained part of its hydration shell. Preference for the D-Val side for complexation could be attributed to the presence of lesser number of side chains and therefore lesser steric hindrance when compared with the L-Val side. The failure to form 'valinomycin- $K^+$ ' like complex was probably due to the larger solution sheath that surrounds  $Ba^{2+}$ .

**At higher valinomycin concentrations:** At concentrations higher than 5 mM, CD studies showed that valinomycin formed, apart from 1:1, 2:1 (peptide sandwich) and 1:2 (ion sandwich) complexes and a 'final complex' (Devarajan and Easwaran, 1981). Figure 4 shows the titration graph obtained by the addition of barium perchlorate to

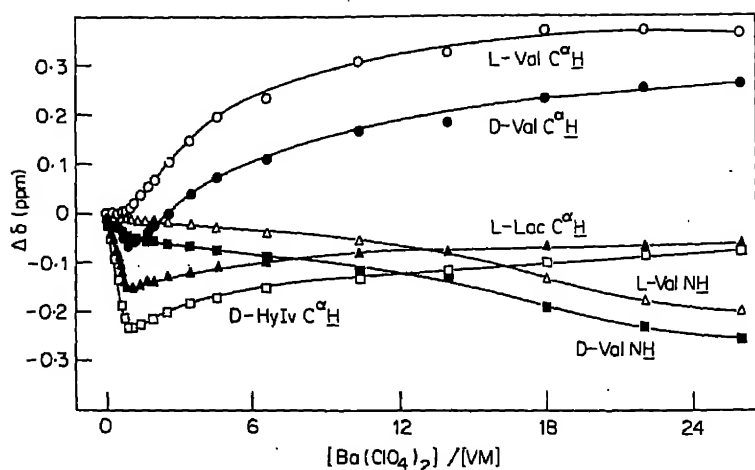


Figure 4. Chemical shift changes at a higher valinomycin concentration, on the addition of barium perchlorate salt.  $[VM] \sim 21$  mM.

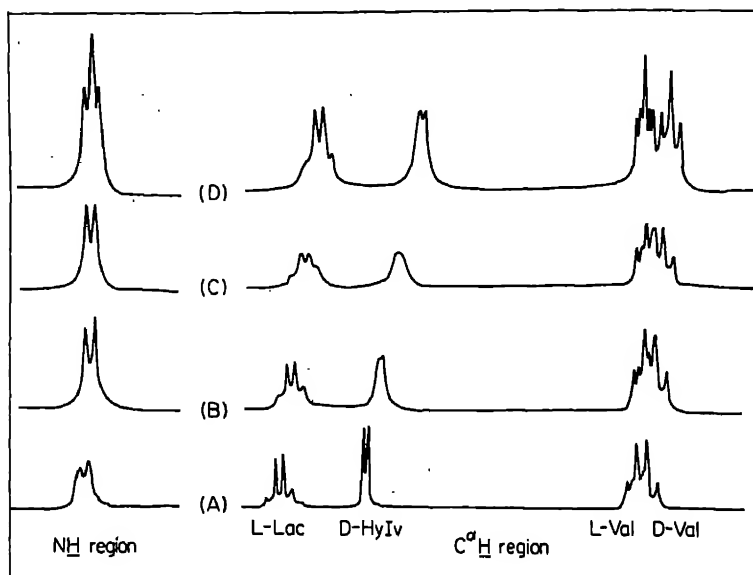
Table 2. Changes in chemical shift values, with barium perchlorate addition, for various protons upto 1:1, and beyond 1:1 until the end of titration. Valinomycin concentration  $\sim 21$  mM (ppm units).

Protons	NH's				C $\alpha$ H's	
State	L-Val	D-Val	L-Lac	D-HyIv	L-Val	D-Val
Upto 1:1	-0.011	-0.049	-0.148	+0.231	0.012	-0.066
Beyond 1:1 till the end	-0.191	-0.205	+0.083	+0.160	0.353	0.330
Total	-0.202	-0.254	-0.065	-0.071	0.365	0.264

valinomycin at  $\sim 21$  mM concentration. A sharp break at 1:1 and a semblance of plateau region at a large excess addition of the barium salt only were seen. The various complexes that are expected to form (2:1 and 1:1 in the beginning stages, and 1:2 and the 'final complex' in the later stages) seemed to be exchanging at a rate faster than NMR time scale and the chemical shift positions therefore represented an average of the various conformations.

Table 2 lists the changes in chemical shift values for the various protons upto 1:1 and beyond 1:1 until the end of the titration. The changes observed up to 1:1 in this titration were less (though similar), when compared with that carried out at 0.37 mM, indicating formation of complexes other than the 1:1 that have opposing influences on the chemical shift changes. The CD studies have indicated that 2:1 complex forms at this stage of titration and has CD contributions opposite to that of 1:1.

Figure 5 represents the NH and the C $\alpha$ H region for a few of the titration points, in the beginning of the experiment. Except L-Val C $\alpha$ H all other lines showed considerable



**Figure 5.** Broadening of the amide and the  $C^\alpha H$  signals except the L-Val  $C^\alpha H$ , in the initial stages of the perchlorate salt addition. The data points correspond to the titration represented in figure 4. The ratios for the data points are (A) 1:0, (B) 1:0.10 (C) 1:0.29 and (D) 1:0.58.

broadening. The absence of any shift (as indicated earlier) or broadening for the L-Val  $C^\alpha H$ , when the 2:1 complex also formed, indicated that the L-Val side was not involved in metal binding in this complex also probably due to steric reasons. The ion was held by six D-Val carbonyl oxygens (Devarajan and Easwaran, 1981).

Thus, by following subtle changes in NMR parameters at a higher valinomycin concentration ( $\sim 21$  mM), it was possible to confirm the formation of the 2:1 complex. Similarly the formation of 1:2 (ion sandwich) is confirmed by following chemical shift changes at a lower temperature ( $-40^\circ\text{C}$ ). Figure 6 shows that all the lines except that of the L-Val  $C^\alpha H$  split into two lines, at  $-40^\circ\text{C}$ , one representing the free and the other, the complex. The lines nearly remain unchanged in their chemical shift positions till 1:1. The complex lines grew, while the free lines decrease in intensity (not shown). The most important difference to be noted with respect to the room temperature titration (figure 4), was the low-field shift of the NH signals beyond 1:1 at low temperature. This observation indicates stabilization of a complex that retained hydrogen bonds—probably the formation of the 1:2 ion sandwich complex, having two barium ions, one on either side of a valinomycin bracelet, held by three ester carbonyls. The other side of the ion would be exposed to interaction with the anions and the solvent molecules.

Determinations of conformations based on NMR parameters was not easy, when these parameters represent an average of many conformations that were exchanging very rapidly among themselves, with no single predominant conformation. Because of this reason, exact conformational characterisation of the various intermediate complexes was not possible. However, at the very end of the titration (as in figure 4), there was an induction of a plateau region, possibly representing the predominant formation

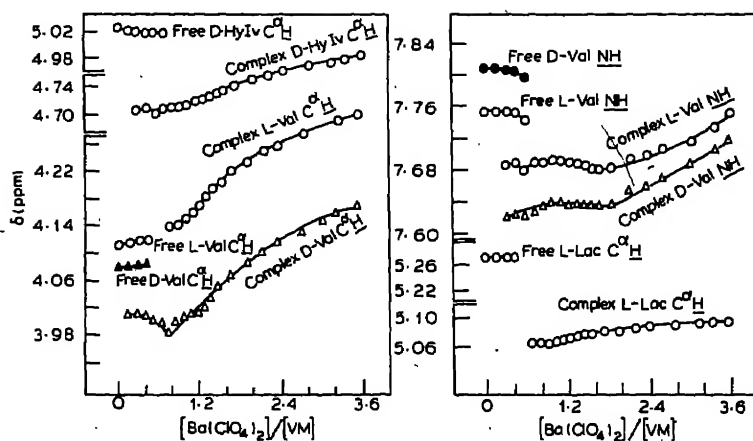


Figure 6. A low temperature ( $-40^{\circ}\text{C}$ ) titration with barium perchlorate salt.  $[\text{VM}] \sim 12 \text{ mM}$ . Splitting of all lines except that of L-Val  $\text{C}^{\alpha}\text{H}$  can be seen. Free lines disappear at 1:1.

of the 'final complex' as reported previously (Devarajan *et al.*, 1980; Devarajan and Easwaran, 1981). Conformational characterisation of this complex will be reported in the latter part of this paper.

#### Studies on valinomycin-barium thiocyanate complexes

**At lower valinomycin concentration:** With barium thiocyanate salt, valinomycin at low concentrations (0.37 mM) formed a 1:1 complex involving the sterically less hindered D-Val side, as with the perchlorate salt. However, unlike in the perchlorate case, the complex was not stable even at this low valinomycin concentration as indicated by the lack of plateau region for the L- and D-Val  $\text{C}^{\alpha}\text{H}$  signals beyond 1:1.

**At higher valinomycin concentration:** At a higher valinomycin concentration ( $\sim 3 \text{ mM}$ ), even the L-Val  $\text{C}^{\alpha}\text{H}$  begins to show changes in the chemical shift position, just from the beginning of barium thiocyanate addition (figure 7). This was in contrast to the titration corresponding to barium perchlorate addition (figure 4). Also, a plateau region beyond 1:2, which was not obtained unless a large excess of the perchlorate salt was added, was easily discerned just beyond 1:2 in the thiocyanate case. While the changes in the L-Val  $\text{C}^{\alpha}\text{H}$  from the beginning of the thiocyanate salt addition indicated the formation of a L-Val side involved complex, the faster approach of the plateau region beyond 1:2 indicates a facilitated formation of the 'final complex' in the presence of the thiocyanate anion.

It can be seen below that the L-Val side involved valinomycin barium thiocyanate complex, that forms in the beginning of the thiocyanate salt addition, has to be a 1:1 complex and definitely not a 2:1 complex. This complex was not formed upon the addition of the perchlorate salt and therefore must involve the linear thiocyanate anions for its formation. The formation of the 2:1 complex does not involve anions or

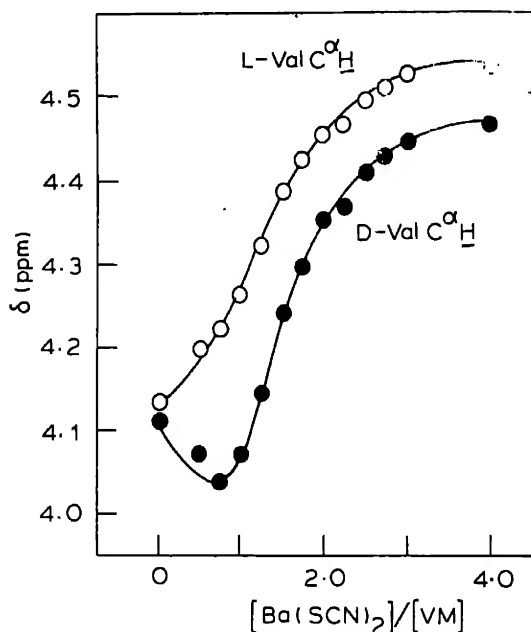


Figure 7. Chemical shift changes at a valinomycin concentration of  $\sim 3$  mM, with barium thiocyanate salt addition, at L- and D-Val C<sup>α</sup>H region.

solvent molecules, while the formation of the 1 : 1 complex is definitely aided by anions and solvent molecules. Because of the involvement of the sterically crowded L-Val side for the complex formation, the superior liganding capacity of the linear anion (thiocyanate) might be needed to stabilise this complex.

#### Carbon-13 studies

Because of the lower natural abundance (1.1 %) and the lesser inherent sensitivity of carbon-13 with respect to proton (Deslauriers and Smith, 1980), there is always a necessity to use a larger concentration of the sample to obtain a good <sup>13</sup>C spectrum. Therefore, the interesting observations made in proton NMR at lower valinomycin concentrations (like at  $\sim 0.4$  mM), could not be confirmed by carbon-13 studies. The low field shift of carbonyl carbons in metal binding (Bystrov *et al.*, 1977), was exploited by following the chemical shift changes of these carbons to supplement the conformational characterisations of the complexes using proton NMR studies.

*On valinomycin-barium perchlorate complexes:* Figure 8 represents the chemical shift changes at the carbonyl carbon region of valinomycin, upon the addition of barium perchlorate. The titration graph clearly showed the break at 1 : 1, and stabilisation at 1 : 2. The high-field shift in the L-Val <sup>13</sup>C' signal, in the initial stages of salt addition,



confirmed the non-participation of L-Val side in metal binding probably due to steric

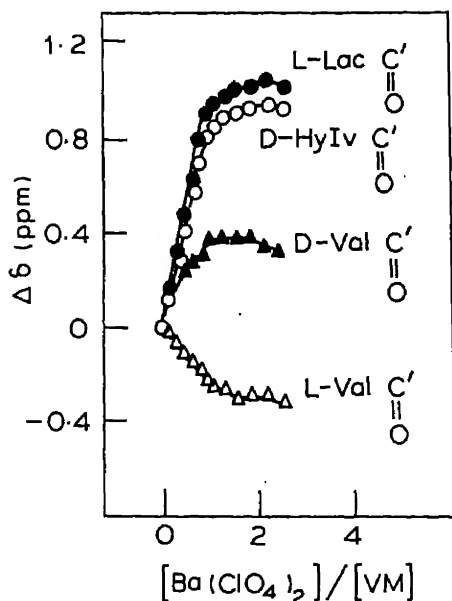


Figure 8. A  $^{13}\text{C}$  NMR titration, representing chemical shift changes at the carbonyl region of valinomycin, with the addition of barium perchlorate salt.

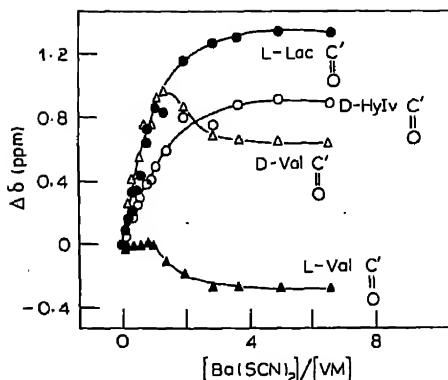


Figure 9. A  $^{13}\text{C}$  NMR titration, representing chemical shift changes at the carbonyl region of valinomycin, with the addition of barium thiocyanate salt.

reasons. The low-field shifting of D-Val, L-Lac and D-HyIv  $^{13}\text{C}'$ s confirmed the

participation of all these carbonyls from the D-Val side in metal binding and provided a possible explanation for the weakening of hydrogen bonding scheme. When a large excess addition of the perchlorate was added (1:13, not shown in figure 8, i.e. the 'final complex' is expected to form), L-Lac and D-HyIv  $^{13}\text{C}'$ s remained at low field

( $\Delta\delta = 1.29$  and  $1.37$  ppm respectively) indicating disengagement of the D-Val  $^{13}\text{C}'$

from metal binding. Therefore, the 'final complex' seemed to involve only amide carbonyls for metal binding unlike all other valinomycin-metal complexes so far reported in the literature (Ovchinnikov *et al.*, 1974; Bystrov *et al.*, 1977).

*On valinomycin-barium thiocyanate complexes:* Figure 9 represents the chemical shift changes at the carbonyl carbon region of valinomycin, on the addition of barium thiocyanate salt. The break shown at 1:1 by the L-Val  $^{13}\text{C}'$  and the stabilisation of the

chemical shift changes of all the signals just beyond 1:2 confirmed the formation of 1:1 and 1:2 respectively.

The proton studies indicated that an L-Val side involved 1:1 complex was also formed in the presence of the thiocyanate anion. However, the L-Val  $^{13}\text{C}'$  did not shift



low field even to a limited extent contrary to expectations. It could be seen from literature (Bystrov *et al.*, 1977) that when there was opening of the bracelet conformation, the L- and D-Val  $^{13}\text{C}'$ 's shifted high-field. Therefore, it is possible that in



the initial stages of the thiocyanate salt addition, the low-field shift of the L-Val  $^{13}\text{C}'$



expected with the formation of the L-Val side involved 1:1 complex was compensated with the high-field shift of the above signal caused by the formation of a small amount of the 'final complex' with its open conformation. It is interesting to note at this point that in the corresponding  $^{13}\text{C}$  titration performed with the perchlorate, the L-Val  $^{13}\text{C}'$

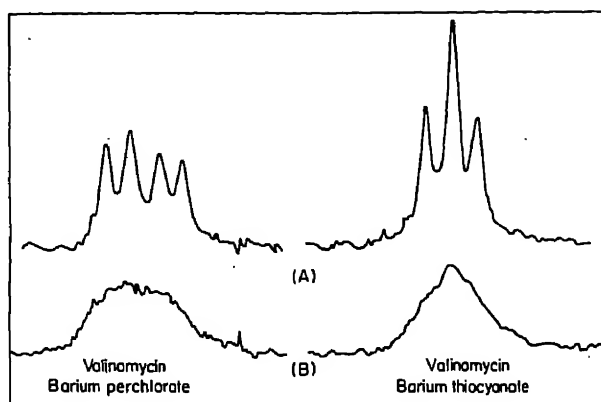


moves high-field from the beginning of the salt addition. The chemical shift changes in the carbonyl region beyond 1:1 in the present titration (figure 9) are similar to those in the perchlorate case (figure 8), confirming the formation of a 'final complex' with 1:2 stoichiometry.

### Conformation of the final complex

*From solution studies:* In our earlier paper dealing with CD studies (Devarajan and Easwaran, 1981), the complex that was formed near the end of the barium salt addition was termed as the 'final complex'. From  $^{13}\text{C}$  NMR studies (see above), it was clear that this complex had 1:2 stoichiometry and the amide carbonyls were involved in metal binding. The inference from the CD studies that this complex may have an open conformation was confirmed by the shift of NH to highfield near the end of barium salt addition to valinomycin (figure 4) and also from a free radical experiment as shown in figure 10. This experiment was conducted at identical concentrations for both valinomycin and the free radical as in the case of uncomplexed valinomycin and its  $\text{K}^+$  complex (see figure 1). While there was no broadening of the amide protons in the case of the both free valinomycin and its  $\text{K}^+$  complex (figure 1), noticeable broadening for these protons was observed in the case of the 'final complex' (figure 10).

The coupling constants that are measured for the 'final complex' and the probable  $\phi$  values corresponding to these constants are listed in table 3. Model building studies clearly ruled out conformations with  $\phi$  values  $+40^\circ$  and  $+80^\circ$  for L-Val ( $-40^\circ$  and  $-80^\circ$  for D-Val), but allowed the other two conformational angles. It can be seen from energy maps corresponding to the various residues present in the valinomycin molecule (Ovchinnikov and Ivanov, 1974; figure 11) that, although at one  $\psi$  value or the other, each of the four  $\phi$  values mentioned above become allowed, it is only around  $\phi = -80^\circ$  for L-Val ( $+80^\circ$  for D-Val), the global minimum energy is found. Also, only at this region of the energy map, large changes in the conformational angles are allowed with only a small increase in energy. For example, the  $\phi$  values can be changed by  $\pm 50^\circ$  with



**Figure 10.** Effect of free radical addition on the 'final complexes' of the barium salts, with valinomycin. Valinomycin concentration is  $\sim 8.9$  mM in both the systems. **A.** Before addition. **B.** After addition.

**Table 3.** Coupling constants measured and the corresponding dihedral angles for the 'final complex'.

Residue	$^3J_{HNC^*H}$	$\phi$ values
L-Val	6.3 Hz	$-160^\circ$ , $-80^\circ$ , $+40^\circ$ , $+80^\circ$
D-Val	6.3 Hz	$160^\circ$ , $80^\circ$ , $-40^\circ$ , $-80^\circ$

only 1 or 2 kcal increase in energy. Since the 'final complex' has an open conformation, it may have a lot of flexibility and  $\phi$  values  $-80^\circ$  for L-Val and  $+80^\circ$  for D-Val seem to be ideal for accommodating this flexibility.

**Comparison with the conformation in the solid state:** In the single crystal studies of valinomycin barium perchlorate complex (Devarajan *et al.*, 1980), it has been found that valinomycin molecule was in an open conformation (with no hydrogen bonds) and held two cations by its amide carbonyls (figure 12). Thus in all major aspects, the conformation observed in the solid state was very similar to that in the 'final complex'. However, while the NMR studies on the 'final complex' have constantly shown a conformer that is  $C_3$  symmetric in NMR time scale, the solid state conformer is non  $C_3$  symmetric. Possibly if there are a good number of near equi-energy conformational states in solution that are non  $C_3$  symmetric in nature, a fast exchange (in NMR time scale), among these conformers may result in the  $C_3$  symmetry observed in NMR.

#### *Exchange among near equi-energy states*

Figure 11 shows the conformational energy maps of the four individual residues L-Val, D-HyIv, D-Val and L-Lac. The conformational angles observed in the solid state and their mean values are shown in figure 4. Table 4 lists all the mean values and the maximum deviations of the observed values from these mean values on either side, for



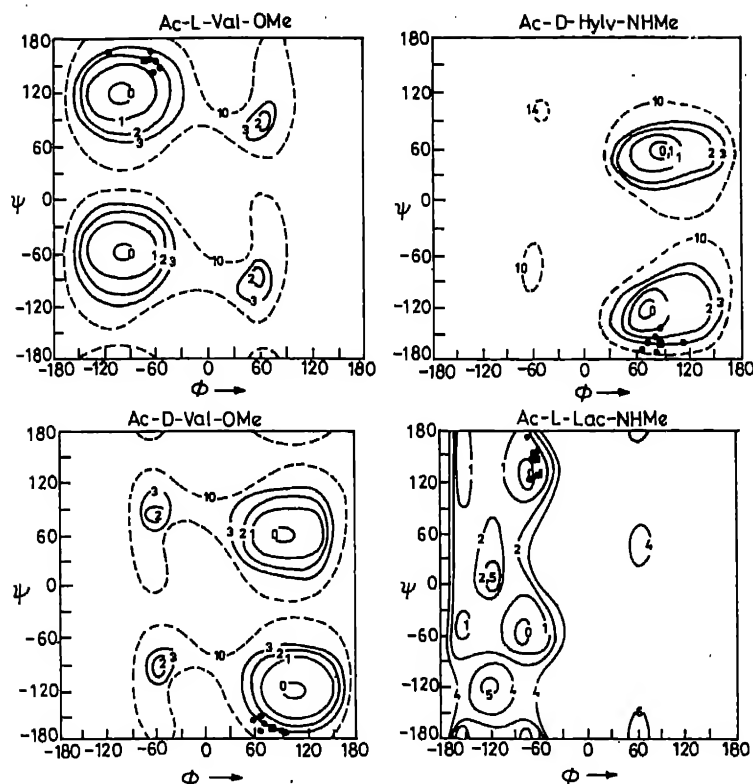
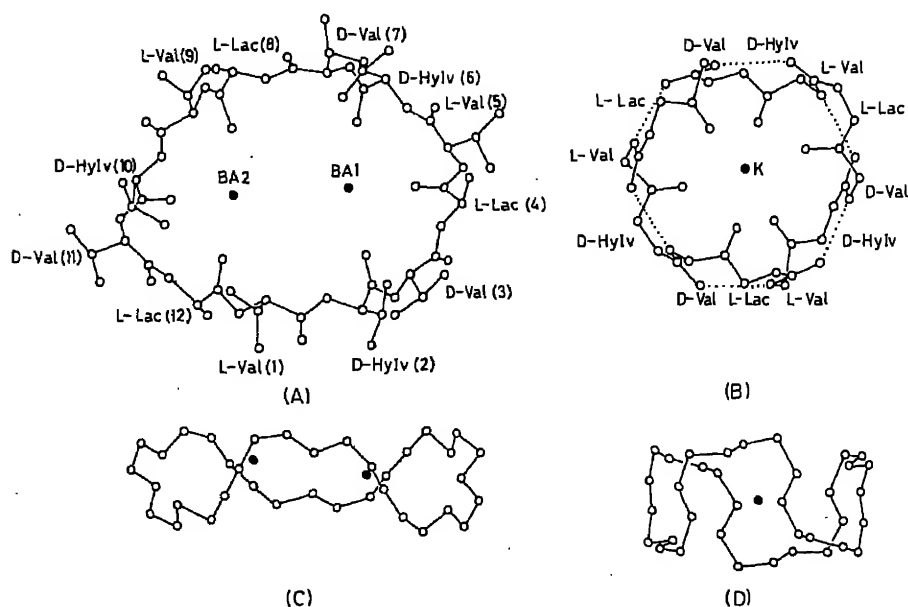


Figure 11. Conformational energy maps for the various residues present in valinomycin (taken from Ovchinnikov and Ivanov, 1974).

all the four individual residues. The deviations from the mean values were less than  $30^\circ$  except for the  $\phi$  value of one L-Val residue which deviated by about  $44^\circ$ . It can be seen from figure 11 that the  $\phi$  value assumed by this residue ( $-121^\circ$ ) falls on an energy contour that shows the same energy at the  $\phi$  value ( $-77^\circ$ ) that is mean for all the L-Val residues. Therefore there will not be any increase in conformational energy despite such a large deviation from the mean.

It can be seen from figure 11 that, for a residue, the conformational changes on either side of the mean ( $\pm 30^\circ$ ) can be achieved by 3 or 4 kcal gain or loss of energy. In the molecule, conformational changes can occur such that the gain in energy at one residue can compensate for the loss at another residue. Apart from such equi-energy states, there can be many other conformational states which differ just by 1 or 2 kcal change in energy. Assuming that the mean conformational angles represented in table 4 correspond to an average conformation in solution, all the conformational angles that are observed for a residue (Devarajan, 1982) on either side of this calculated mean value can be achieved with a minimum change in the conformational energy. As the energy separation among these various conformational states is not high, the populations that occupy each individual conformational state may be very close, and the exchange among these also may be fast, leaving the molecule to achieve  $C_3$  symmetry on an



**Figure 12.** 'Top' view of the valinomycin molecule in its (A) barium perchlorate complex (Devarajan *et al.*, 1983) and (B) Potassium iodide complex (Neupert-Laves and Dobler, 1975). C and D. Represent the corresponding 'side' views, with the side chain atoms and carbonyl oxygens omitted for clarity. The residue numbering is indicated in (A).

**Table 4.** Mean values and the deviations on either side from the mean values for the various conformational angles (degrees) observed in the two valinomycin-barium structures solved in the solid state (Max = maximum value, Min = minimum value; Devarajan, 1982).

Residue	L-Val			D-HyIv			D-Val			L-Lac		
Angle	$\phi$	$\psi$	$\omega$	$\phi$	$\psi$	$\omega$	$\phi$	$\psi$	$\omega$	$\phi$	$\psi$	$\omega$
Mean	-77	157	-179	82	-153	-177	73	-162	-178	-67	149	174
(Max-Mean)	17	10	3	28	17	5	19	16	6	7	22	7
(Mean-Min)	44	15	6	16	19	6	16	13	12	8	21	9

average, from many near equi-energy non  $C_3$  symmetric conformational states. Thus the 'final complex' obtained in solution on an average may resemble the conformation observed in the solid state.

## Conclusions

It is known that barium ion interferes with physiological functions involving specific binding of  $K^+$  ion, because of its similar size (Armstrong and Taylor, 1980). Due to its

higher charge density and the resultant larger solvation sheath, the double charged barium ion shows a tendency to retain part of its solvation sheath in almost all the complex structures (Johnson *et al.*, 1970; Metz *et al.*, 1971; McClelland, 1974; Raston and White, 1976; Hughes *et al.*, 1978). This tendency was found even in situations where  $K^+$  did not retain any solvent molecule under identical conditions (Metz *et al.*, 1971). This could be one of the reasons why the 'final complex' and the two barium complexes solved in the solid state have a flat conformation probably to allow the alkaline earth metal to be accessible to anions and solvent molecules from both sides.

The 'final complex' of valinomycin-barium system has been unambiguously characterized as an open conformation. This can serve as a model for valinomycin conformation in polar solvents. We have also confirmed the formation of both peptide as well as ion sandwich complexes apart from two peripherally-ion-bound 1:1 complexes. While the open conformation may not serve any useful role to explain the transport process, the peripherally-ion-bound 1:1 complexes definitely aid in explaining the complexation mechanism at the membrane interface and the sandwich complexes help in understanding a modified carrier system involving more than one carrier (a relay mechanism) and indicate the possibility of ion-transport by a channel mechanism involving a column of stacked carriers.

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## Studies on *Aspergillus niger* glutamine synthetase: Regulation of enzyme levels by nitrogen sources and identification of active site residues

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**Abstract.** The specific activity of glutamine synthetase (L-glutamate: ammonia ligase, EC 6.3.1.2) in surface grown *Aspergillus niger* was increased 3–5 fold when grown on L-glutamate or potassium nitrate, compared to the activity obtained on ammonium chloride. The levels of glutamine synthetase was regulated by the availability of nitrogen source like  $\text{NH}_4^+$ , and further, the enzyme is repressed by increasing concentrations of  $\text{NH}_4^+$ . In contrast to other micro-organisms, the *Aspergillus niger* enzyme was neither specifically inactivated by  $\text{NH}_4^+$  or L-glutamine nor regulated by covalent modification.

Glutamine synthetase from *Aspergillus niger* was purified to homogeneity. The native enzyme is octameric with a molecular weight of  $385,000 \pm 25,000$ . The enzyme also catalyses  $\text{Mn}^{2+}$  or  $\text{Mg}^{2+}$ -dependent synthetase and  $\text{Mn}^{2+}$ -dependent transferase activity.

*Aspergillus niger* glutamine synthetase was completely inactivated by two mol of phenylglyoxal and one mol of N-ethylmaleimide with second order rate constants of  $3.8 \text{ M}^{-1} \text{ min}^{-1}$  and  $760 \text{ M}^{-1} \text{ min}^{-1}$  respectively. Ligands like Mg. ATP, Mg. ADP, Mg. AMP, L-glutamate  $\text{NH}_4^+$ ,  $\text{Mn}^{2+}$  protected the enzyme against inactivation. The pattern of inactivation and protection afforded by different ligands against N-ethylmaleimide and phenylglyoxal was remarkably similar. These results suggest that metal ATP complex acts as a substrate and interacts with an arginine residue at the active site. Further, the metal ion and the free nucleotide probably interact at other sites on the enzyme affecting the catalytic activity.

**Keywords.** *Aspergillus niger*; glutamine synthetase; nitrogen regulation; purification; kinetic properties; active site residues.

### Introduction

Glutamine functions as a nitrogen donor for a number of nitrogenous end products. Glutamine synthetase catalyzing the formation of L-glutamine has been established to be a key regulatory enzyme in several Gram negative bacteria (Stadtman and Ginsburg, 1974; Tyler, 1978). Although a few studies on this enzyme have been carried out with eukaryotic micro-organisms (Limon-Lason *et al.*, 1977; Quinto *et al.*, 1977; Mora *et al.*, 1980; Ferguson and Sims, 1974a; Generalova and Abramova, 1975; Legrain *et al.*, 1982), not much is known on the mechanisms of regulation of this enzyme activity.

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Abbreviations used: DEAE, Diethylaminoethyl; Buffer A, 20 mM imidazole-HCl buffer, pH 7.5 containing 0.1 mM EDTA, 2 mM 2-mercaptoethanol and 5% glycerol; Glu, L-glutamate;  $\gamma$ GHA,  $\gamma$ -glutamylhydroxamate; NEM, N-ethylmaleimide; pHMB, *p*-hydroxymercuribenzoate; Gln, L-glutamine; EDC, 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide; SDS, sodium dodecyl sulphate; Tris, Tris(hydroxymethyl)aminomethane,  $M_r$ , molecular weight;  $k_{app}$ , pseudo first order rate constant.

*Aspergillus niger* is extensively used in the manufacture of citric acid by fermentation. This derangement of carbon metabolism has been attributed to an alteration in the activities and synthesis of the enzymes of tricarboxylic acid cycle (Kubicek and Rohr, 1978; Bowes and Matthey, 1980). However these results do not completely explain the physiology of citric acid fermentation. For the following reasons, we hypothesize that a marked decrease in the activity of glutamine synthetase under conditions favourable for citric acid excretion, would result in shutting down of the nitrogen metabolism and consequently backing up of the carbon metabolism at the tricarboxylic acid cycle level: (a) Glutamine synthetase via glutamate dehydrogenase links tricarboxylic acid cycle and nitrogen metabolism; (b) Conditions used for maximal citric acid production are, poor buffering and high acidity of the medium, situations which are unfavourable for optimal activity of enzymes; (c) Manganese deficiency is an essential prerequisite for optimal citric acid production and glutamine synthetase is one of a few enzymes requiring manganese. As an initial step in testing this hypothesis, a study of the regulation of the enzyme levels and its activity in *A. niger* grown in different nitrogen sources was undertaken.

## Materials and methods

All the chemicals used were of analytical grade or purchased from Sigma Chemical Company, St. Louis, Missouri, USA, except DEAE-Sephacel and Sepharose-4B which were from Pharmacia Fine Chemicals, Uppsala, Sweden; Biogel A5M was from Biorad Laboratories, Richmond, California, USA.

### Organism and growth conditions

*A. niger* (UBC 814) was grown as surface cultures in one liter flasks containing 100 ml of the culture medium. The medium composition was essentially the same as described for *A. nidulans* by Pateman (1969) with minor modifications. Addition of micronutrients (mg/litre);  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 20;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 10;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 3;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1.0; improved the growth and yield of cells. One per cent glucose was used as carbon source. Nitrogenous compounds were used at 100 mM concentration, unless otherwise mentioned.

Uniform spore suspension (3 ml) obtained from 7–10 day old cultures was used as inoculum. The cells were harvested in the maximal growth phase (45th after inoculation), washed and stored at  $-20^\circ\text{C}$ .

### Preparation of cell extracts

Frozen fungal mat was crushed with equal amounts (wt/wt) of fine glass powder and extracted with 5 vol of 20 mM 2-mercaptoethanol and 5% glycerol (Buffer A). The clear supernatant obtained after centrifugation at 12,000 *g* for 20 min in a Sorvall RC-5B refrigerated centrifuge, was designated as the crude extract. All the operations were carried out at 0–4°C.

### Assay of glutamine synthetase

The enzyme was assayed by the colourimetric determination of  $\gamma$ -glutamylhydroxamate ( $\gamma$ GHA) formed (Lipmann and Tuttle, 1945). The reaction mixture (0.5 ml) for estimating  $Mg^{2+}$ -dependent synthetase activity contained; 100 mM monosodium L-glutamate (Glu), 50 mM hydroxylamine-HCl (freshly neutralized with 1 M NaOH), 10 mM ATP, 20 mM  $MgCl_2$  and 100 mM imidazole-HCl buffer, pH 7.8. The components for the  $Mn^{2+}$ -dependent synthetase activity of the enzyme were same as those for the  $Mg^{2+}$ -dependent activity except that 4 mM  $MnSO_4$  replaced 20 mM  $MgCl_2$  and was assayed at the optimum pH of 5.5.

The reaction mixtures (0.5 ml) for the assay of  $Mn^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity contained; 112.5 mM L-glutamine (Gln), 50 mM hydroxylamine-HCl, 20 mM sodium arsenate, 1 mM  $MnSO_4$ , 0.5 mM ADP and 100 mM imidazole-HCl buffer pH 6.0.

The reaction was started by the addition of an appropriate amount of enzyme and incubated for 15 min at 28°C. The amount of  $\gamma$ GHA formed was estimated (Rowe *et al.*, 1970). One unit of enzyme was defined as the amount that catalyzed the formation of one  $\mu$ mol of  $\gamma$ GHA per min. Specific activity was expressed as units/mg protein. Protein was estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

### Preparation of AMP-Sepharose

Aminohexane-Sepharose prepared as described by March *et al.* (1974), was washed thoroughly with glass distilled water. Disodium salt of AMP (1 mmol) was pre-incubated with 2.5 mmol of 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide (EDC) for 10 min at 60°C in 3 ml of distilled water and then added to a stirred suspension of (10 ml packed volume) the gel and 7 ml of distilled water. The reaction mixture was stirred gently for 18 h at room temperature and for a further 24 h at 0–4°C. At the end of the reaction, the gel was washed with water and stored at 4°C. The incorporation of the ligand attached by a phosphoramidate linkage was measured by the method of Failla and Santi (1973).

### Chemical modification of amino acid residues at the catalytic site of *A. niger* glutamine synthetase

For N-ethylmaleimide (NEM) inactivation, the enzyme was made essentially free of 2-mercaptoethanol by passing it through a G-25 column (1  $\times$  5 cm) just before use. The inactivation of the enzyme in 50 mM imidazole-HCl buffer, pH 7.5 by either phenylglyoxal or NEM was carried out at 28°C by incubating the enzyme (40–60  $\mu$ g) with appropriate concentrations of the reagent in a scaled up reaction mixture (500  $\mu$ l) and withdrawing aliquots (50  $\mu$ l) at regular time intervals indicated. The reaction was terminated by diluting the enzyme into assay mixture directly. It was ensured that the chemical modification reaction was not occurring during the time required for estimating the enzyme activity. The velocity of the enzyme catalyzed reaction at zero

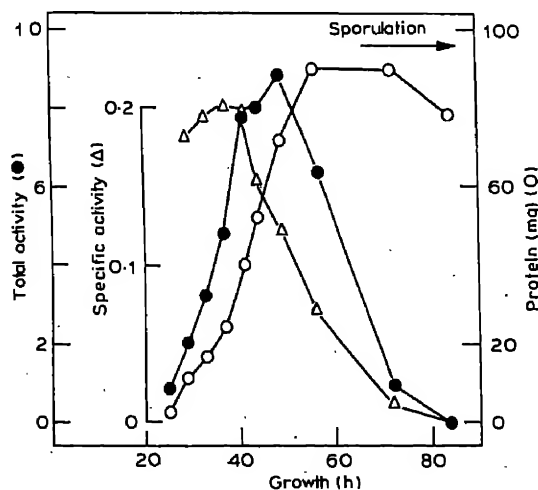
time i.e., immediately after the addition of inactivating reagent, was normalized to 100 and the residual activity was expressed as per cent of this normalized value. During inactivation of glutamine synthetase by either phenylglyoxal or NEM, there was a parallel loss of both  $\text{Mg}^{2+}$ -dependent synthetase and  $\text{Mn}^{2+}$ -dependent transferase activity and the protective influence of various ligands on the two activities was identical for both the inactivations. Hence, the enzyme activity was conveniently monitored by  $\gamma$ -glutamyl transferase assay. The effect of various ligands on modification was checked by including them in the inactivating system at appropriate concentrations.

## Results

### *Correlation between growth on different nitrogen sources and glutamine synthetase levels*

Figure 1 shows the growth curve for Glu-grown (100 mM) *A. niger*. Both specific and total activities of glutamine synthetase were highest around the maximal growth phase (45 h). A rapid decline in the levels of this enzyme occurred just before the onset of conidiation. The ratio of  $\text{Mg}^{2+}$ -dependent glutamine synthetase to  $\gamma$ -glutamyl transferase activity remained constant (0.12–0.18) during different stages of growth. Similar pattern of growth and changes in enzyme activity were obtained with L-glutamine (Gln), potassium nitrate and  $\text{NH}_4^+$ . However, with nitrate the yield of cells was decreased by about 50%.

When Glu (100 mM) or  $\text{NO}_3^-$  (100 mM) was used as the nitrogen source, the specific



**Figure 1.** Growth curve and the levels of glutamine synthetase in *A. niger* grown on 100 mM Glu. The cells were harvested at time intervals indicated and the protein content, (O); total (●) and specific (Δ)  $\text{Mg}^{2+}$ -dependent glutamine synthetase activity in the crude extracts prepared as described in materials and methods is presented. Similar results were obtained when the  $\text{Mn}^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity was monitored.



activity of the enzyme at 45 h was 0.096 and 0.135 respectively, compared to the values in the range 0.023–0.036 obtained when Gln (50 mM), L-asparagine (50 mM), urea (50 mM) and  $\text{NH}_4^+$  (100 mM), were used as the nitrogen sources. In all the cases, increasing the concentration of nitrogen source from 50 mM to 100 mM resulted in a decrease in the specific activity of the enzyme. The ratio of  $\text{Mg}^{2+}$ -dependent glutamine synthetase to  $\gamma$ -glutamyl transferase activity remained essentially constant (0.14–0.12) irrespective of the nature of the nitrogen source. In order to determine whether Glu was specifically inducing the enzyme or not, the levels of this enzyme were monitored in cells grown on media containing a mixture of nitrogenous compounds (table 1). Even under conditions for the enzyme induction, *e.g.*, Glu (100 mM), addition of a small amount of a second, preferred nitrogen source for *e.g.*, Gln (10 mM) or  $\text{NH}_4^+$  (10 mM) decreased the activity of the enzyme to near basal levels. Similar results were obtained with  $\text{NO}_3^-$  grown cells (data not presented), but the concentration of Gln or  $\text{NH}_4^+$  (30 mM) required were slightly higher. A direct experiment to check the effect of varying concentrations of  $\text{NH}_4^+$  on the levels of this enzyme was carried out (table 2). The concentration of the nitrogen source had an inverse relationship with the levels of

Table 1. The effect of a second nitrogen source on the glutamine synthetase levels in *A. niger* grown on Glu.

Nitrogen Source (100 mM Glu)	Specific <sup>a</sup> activity	Ratio <sup>b</sup>
Control	0.097	0.16
+ $\text{NH}_4\text{Cl}$ (10 mM)	0.046	0.16
+ $\text{KNO}_3$ (10 mM)	0.083	0.16
+L-Glutamine (10 mM)	0.045	0.14
$\text{NH}_4\text{Cl}$ alone (100 mM)	0.027	0.16
Methylamine alone (100 mM)	0.051	0.16

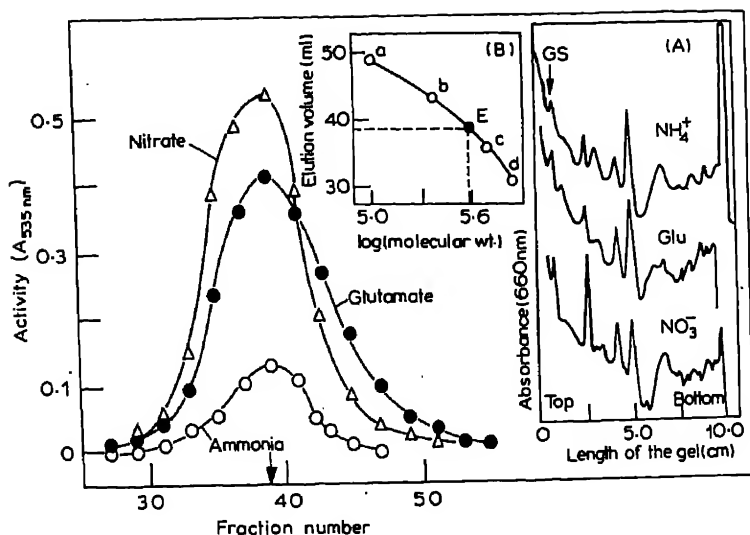
<sup>a</sup>  $\text{Mg}^{2+}$ -dependent glutamine synthetase activity, units/mg protein.

<sup>b</sup> Ratio of  $\text{Mg}^{2+}$ -dependent glutamine synthetase to  $\text{Mn}^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity.

Table 2. Regulation of glutamine synthetase levels by  $\text{NH}_4^+$  concentration in the medium.

$\text{NH}_4\text{Cl}$ (mM)	Specific activity*
10	0.059
30	0.051
50	0.037
70	0.029
90	0.026
100	0.027

\*  $\text{Mg}^{2+}$ -dependent glutamine synthetase activity.



**Figure 2.** Biogel A5M gel filtration profiles of glutamine synthetase in crude extracts of *A. niger* grown on  $\text{NH}_4^+$ , Glu and  $\text{NO}_3^-$ . The arrow indicates peak position.

**A.** Analytical polyacrylamide gel electrophoresis of crude glutamine synthetase. The crude extracts (200  $\mu\text{g}$  protein) obtained from cells grown on  $\text{NH}_4^+$ , Glu or  $\text{NO}_3^-$  were subjected to analytical polyacrylamide gel electrophoresis at  $4^\circ\text{C}$ , in 7.5% polyacrylamide gels in 0.5 M Tris-0.39 M glycine buffer, pH 8.6, at a constant current of 2 mA per tube for 3 h (Davis, 1964). One set of gels were stained for protein using Coomassie brilliant blue G (0.02% in 3.5% perchloric acid) and a second set of gels were sliced into 0.5 mm bits and the protein was extracted into the assay buffer by maceration and enzyme activity was measured. The stained gels were scanned at 660 nm in a Beckmann Model-26 Spectrophotometer. The arrow indicates the position of glutamine synthetase activity.

**B.** The  $M_r$  of the native enzyme was determined by the method of Andrews (1965), using Biogel A5M column (0.6  $\times$  65 cm) equilibrated with buffer A and operated at a flow rate of 10–12 ml/h. The marker proteins (2–5 mg each, in a total volume of 0.5 ml) were loaded separately and 0.75 ml fractions were collected. The elution of hexokinase (a, 102,000), catalase (b, 232,000), ferritin (c, 480,000) and thyroglobulin (d, 669,000) were monitored by measuring the absorbance at 280 nm. The numbers in parenthesis indicate  $M_r$  of markers. Glutamine synthetase (E) was chromatographed separately and its elution profile was monitored by assaying the  $\text{Mn}^{2+}$ -dependent transferase activity. The  $M_r$  of the enzyme was calculated by extrapolation to be  $385,000 \pm 25,000$ . Both the purified enzyme (150  $\mu\text{g}$ ) and the enzyme present in the crude extracts were extruded from the column at identical volumes.

glutamine synthetase. Higher the concentration of nitrogen source, lower was the glutamine synthetase specific activity. Similar results were obtained with urea, Gln, Glu, L-asparagine, and  $\text{NO}_3^-$ .

It was observed that the molecular and catalytic properties of glutamine synthetases were dependent on the nitrogen source in the growth medium (Vichido *et al.*, 1978; Sims *et al.*, 1974b). In order to examine whether any such changes were occurring in the case of *A. niger* enzyme, the organism was grown on Glu,  $\text{NH}_4^+$  or  $\text{NO}_3^-$  (all at 100 mM) and the activity of the enzyme in the crude extracts as well as its electrophoretic mobility (figure 2A) and elution profile on gel filtration (figure 2) was determined. The intensities

of the dye stain corresponding to glutamine synthetase protein were roughly proportional to the activity measured in the crude extracts. However, the mobility of glutamine synthetase in all the three cases was similar. Also, the enzyme from these cases eluted from the gel filtration column (Biogel A5M,  $0.6 \times 65$  cm) as a single symmetrical peak with same elution volume corresponding to a molecular weight ( $M_r$ ) of  $385,000 \pm 25,000$  (figure 2B).

#### Absence of regulation by rapid inactivation

In order to determine whether the modulation of enzyme activity *in vivo* was occurring by enhanced degradation of the enzyme or by its covalent modification as in *Escherichia coli* (Stadtman and Ginsburg, 1974), cells harvested at maximal growth phase (45 h) on Glu were transferred on to Gln (100 mM) or  $\text{NH}_4^+$  (100 mM) medium and glutamine synthetase activity was measured. The activity decreased by only about 50–60% over 4–5 h (figure 3). This suggested that there was no rapid and specific *in vivo* glutamine synthetase inactivating system. Final glutamine synthetase levels to which the organism adjusted were comparable to the basal levels of the enzyme obtained when grown on Gln or  $\text{NH}_4^+$ . The ratio of  $\text{Mg}^{2+}$ -dependent glutamine synthetase to  $\gamma$ -glutamyl transferase activity remained constant (0.14–0.16) at each time point.

When the crude extract from *A. niger* cells grown on Gln and  $\text{NH}_4^+$  were mixed with the extracts of glutamate-grown cells (45 h), incubated upto 30 min at room temperature (28°C) and monitored for glutamine synthetase activity, there was no loss of activity in the combined extracts (data not presented).

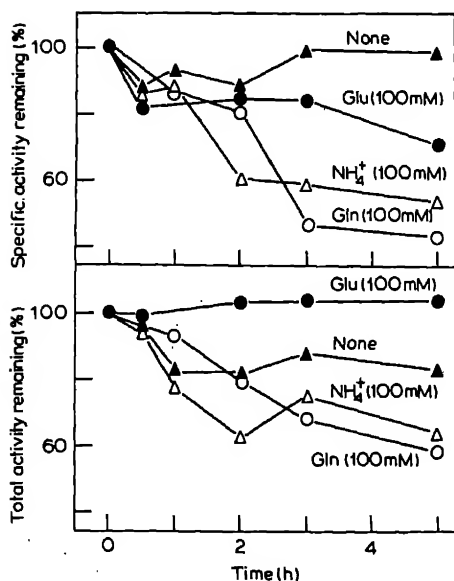


Figure 3. Inactivation of glutamine synthetase *A. niger* was grown on 100 mM Glu for 45 h, harvested, washed aseptically and transferred on to a medium containing no nitrogen source, (Δ); Glu, (●); Gln, (○); and  $\text{NH}_4^+$  (Δ) (all at 100 mM). The total and specific activities were determined at the time intervals indicated after transfer. The 100% values correspond to a  $\gamma$ -glutamyl transferase specific activity of 0.647.

*Purification of glutamine synthetase*

Cells grown on 66 mM Glu as the sole source of nitrogen were used as the starting material. The enzyme was stabilized against inactivation during purification by including 2-mercaptoethanol, EDTA and 5 % glycerol in the buffer (Buffer A) during all the steps of purification. The purification was carried out at 0–4°C. Frozen fungal mat (150 g) crushed with an equal amount (wt/wt) of fine glass powder, extracted with 3 vol of buffer A, was passed through two layers of cheese cloth and the filtrate centrifuged at 12,000 *g* for 20 min in a Sorvall RC-5B refrigerated centrifuge. The supernatant (crude extract, table 3) was treated with protamine sulphate (400 mg), and the precipitated nucleoproteins were removed by centrifugation. To this supernatant, solid ammonium sulphate was added to 45 % saturation and the precipitate formed was discarded. The precipitate obtained on raising the saturation to 60 % was collected, dissolved in a small amount of buffer A and desalted by gel filtration on a Sephadex G-25 column (2 × 30 cm). This fraction (ammonium sulphate fraction, table 3) was loaded on to a DEAE-Sephacel column (2 × 15 cm) equilibrated with buffer A. The column was washed with the same buffer until the eluate absorbance (at 280 nm) was less than 0.05. A linear KCl gradient (0–0.5 M, 200 ml) at a flow rate of 20 ml/h was used to elute the enzyme from the column. Fractions (2 ml) having maximal activity were pooled (DEAE-Sephacel fraction, table 3). The pooled enzyme was diluted with buffer A to a KCl concentration less than 50 mM and was applied on to the AMP-Sepharose column (1.5 × 6 cm) at a flow rate of 12 ml/h. The unadsorbed protein fraction had no enzyme activity. The column was washed with buffer A till all the unadsorbed protein was leached out of the column. The enzyme was eluted from the column with the same buffer containing 5 mM ATP (neutralized to pH 7.0 with 2 M NaOH). Fractions (1 ml) were collected, assayed for enzyme activity and active fractions (specific activity > 15.0) were pooled (AMP-Sepharose enzyme, table 3). The enzyme obtained from the previous step was applied on to a Sepharose-4B column (1.2 × 90 cm) which was previously equilibrated with buffer A. Fractions (1 ml) were collected and assayed for the enzyme activity. Those fractions having more than 50 % activity of the peak fraction were pooled (Sepharose-4B fraction, table 3).

Table 3. Purification of glutamine synthetase from *A. niger*.

Step	Total activity (Units) <sup>a</sup>	Specific activity (Units/mg)	Fold purification	Per cent recovery
Crude	841	0.4	1	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (45–60 %)	631	1.4	3	75
DEAE-Sephacel	457	4.1	10	54
AMP-Sepharose	185	18.7	46	22
Sepharose 4B	67	19.8	49	8

Weight of the fungal mat used = 150 g.

<sup>a</sup>  $\mu$ mol of  $\gamma$ GHA formed/min at 28°C. The  $Mn^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity was monitored.

A summary of the purification procedure is given in table 3. This procedure resulted in a 50-fold purification of the enzyme with 8% recovery. The recoveries and specific activity of the enzyme varied somewhat from preparation to preparation and depended on the fractions that were pooled in the last two steps of purification. The final gel filtration step was essential to remove trace contaminants. The enzyme thus obtained was divided into small volumes and stored at  $-40^{\circ}\text{C}$ . Freshly thawed enzyme was used in each experiment.

#### Physico-chemical and kinetic properties of the enzyme

The final preparation (table 3) showed a single band (figure 4C) on the disc gel electrophoresis (Davis, 1964) in Tris(hydroxymethyl)aminomethane (Tris)-glycine buffer pH 8.6, and the enzyme activity peak coincided well with the protein band observed on staining the gel with Coomassie brilliant blue G (figure 4). From standard curve (figure 2, Inset B) drawn using thyroglobulin (669,000), ferritin (480,000), catalase (232,000) and hexokinase (102,000) as marker proteins, the  $M_r$  of the native, *A. niger* glutamine synthetase was calculated to be  $385,000 \pm 25,000$ . The subunit  $M_r$  of the enzyme was obtained from sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis using bovine serum albumin (67,000), ovalbumin (43,000) and  $\alpha$ -chymotrypsinogen (24,000) as markers (Weber and Osborn, 1969). The enzyme gave a single protein band corresponding to a  $M_r$  of  $53,000 \pm 5,000$  (figure 4, Inset D).

The  $\text{Mn}^{2+}$ - and  $\text{Mg}^{2+}$ -dependent glutamine synthetase activities were linear with enzyme concentration upto  $18\text{ }\mu\text{g}$  and  $10\text{ }\mu\text{g}$ , respectively. The  $\text{Mn}^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity of the enzyme was linear upto  $7\text{ }\mu\text{g}$  protein. All the three activities were linear with time upto 20 min and the enzyme activities were routinely assayed for 15 min. The  $\text{Mg}^{2+}$ -supported glutamine synthetase activity functioned optimally at  $33^{\circ}\text{C}$ . The  $\text{Mg}^{2+}$ -dependent synthetase activity had a pH optimum around 7.8, whereas the  $\text{Mn}^{2+}$ -supported synthetase activity was maximal at pH 5.5 and the

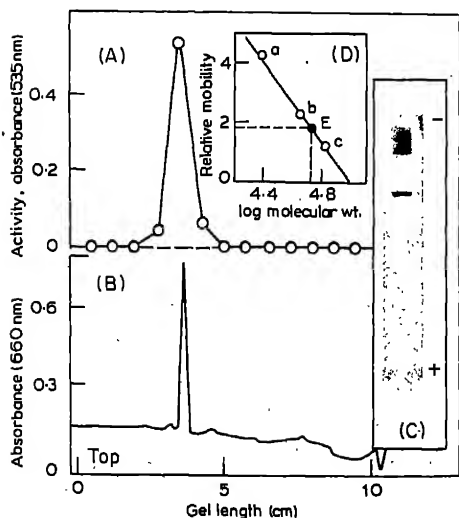


Figure 4. Polyacrylamide gel electrophoresis of the purified *A. niger* glutamine synthetase ( $100\text{ }\mu\text{g}$  of the enzyme used). Details of the procedure are given in legend to figure 2). After electrophoresis the gel was sliced into  $0.5\text{ mm}$  pieces, the enzyme extracted by maceration in buffer A ( $0.2\text{ ml}$ ). The reaction was started by the addition of substrates and  $\gamma$ -glutamyl transferase activity was measured (A). A duplicate gel was stained and the absorbance measured at  $660\text{ nm}$  (B). The gel photograph is shown as inset C. The  $M_r$  was determined by extrapolation from the standard curve (inset D).

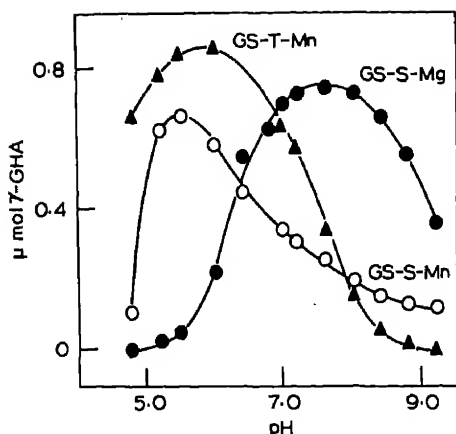


Figure 5. pH Optima of *A. niger* glutamine synthetase activities.

The  $Mg^{2+}$ -dependent synthetase, (●); the  $Mn^{2+}$ -dependent synthetase, (○); and the  $\gamma$ -glutamyl transferase, (Δ), activities were determined using acetic acid/sodium acetate (pH 4.8), Tris, maleate-KOH (pH 5.2–6.0) and Tris-imidazole-HCl (pH 6.0–9.2) buffers (all at 100 mM). The buffer ions had no effect on the enzyme activity. The enzyme concentrations used were usually about 8  $\mu$ g protein for synthetase assays and 3  $\mu$ g for the transferase assay.

$Mn^{2+}$ -dependent  $\gamma$ -glutamyl transferase reaction of the enzyme showed a pH optimum of 6.0 (figure 5).

Glutamine synthetase from *A. niger* could use either  $NH_4^+$  or  $NH_2OH$  as the substrate. The enzyme was about 50 % active when Glu was replaced by D-glutamate in the assay. Of the various metal ions tested ( $Co^{2+}$ ,  $Cr^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Mg^{2+}$  and  $Mn^{2+}$ ) only  $Mn^{2+}$  and  $Mg^{2+}$  were effective as activators of the enzyme. The nucleotide triphosphate requirement of the enzyme was quite specific as CTP, GTP, ITP, TTP and UTP could not replace ATP, in the biosynthetic reaction.

Typical hyperbolic saturation curves (figures not given) were obtained with Glu,  $NH_2OH$  and ATP (at 20 mM  $MgCl_2$ ). At a fixed concentration of Glu (100 mM) the effect of varying concentrations of  $NH_2OH$  (0–50 mM) on the enzyme activity was determined. The maximal velocity was observed at 10 mM  $NH_2OH$ . The effect of varying concentrations of Glu (0–150 mM) on the enzyme activity was studied at a fixed concentration of  $NH_2OH$  (50 mM). The activity was maximal around 100 mM Glu concentration. The concentration of ATP required for maximum enzyme activity was determined by studying the effect of varying concentrations of ATP on the reaction velocity at a constant and saturating concentration of Glu and  $NH_2OH$ . The velocity was highest at a concentration of 10 mM ATP when a 2-fold excess of  $Mg^{2+}$  was used. The  $K_m$  values for the substrates of  $Mg^{2+}$ -dependent synthetase activity viz. Glu,  $NH_2OH$  and ATP were calculated from the linear Lineweaver-Burk plots after Lee-Wilson (1971) modification and are summarized in table 4. The  $Mn^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity of *A. niger* glutamine synthetase also showed hyperbolic saturation patterns with the substrates Gln and  $NH_2OH$  (figures not presented). This activity of the enzyme required optimal concentrations of  $Mn^{2+}$  (1 mM), ADP (0.5 mM) and sodium arsenate (20 mM) as nonconsummable substrates. When  $NH_2OH$  concentration was varied 0–50 mM at a fixed concentration of Gln (112.5 mM), maximal activity was obtained at 30 mM of  $NH_2OH$ . Gln concentration could not be increased beyond 112.5 mM because of the limited solubility of this amino acid in water (saturated solution of Gln in water is about 250 mM). The  $K_m$  values for Gln and  $NH_2OH$  were obtained by the above mentioned method. The Michaelis constants for all the substrates of the enzyme have been summarized in table 4. The

**Table 4.** Michaelis constants for different substrates of glutamine synthetase from *A. niger*.

Substrate	$K_m$ (mM) <sup>a</sup>
<b>Mg<sup>2+</sup>-dependent synthetase activity:</b>	
Glu	10.0
NH <sub>2</sub> OH	0.5
ATP (at 20 mM MgCl <sub>2</sub> )	1.5
<b>Mn<sup>2+</sup>-dependent <math>\gamma</math>-glutamyl transferase activity:</b>	
Gln <sup>b</sup>	235
NH <sub>2</sub> OH	4.0
Mn <sup>2+</sup> ( $K_{0.5}$ at 0.5 mM ADP)	0.07

<sup>a</sup> The Michaelis constants for various substrates were obtained by applying Lee-Wilson (1971) modification to the double reciprocal plots. This was necessary because, the  $K_m$  value for NH<sub>2</sub>OH and Mg.ATP were low and hence appreciable amounts of product formation (> 10%) could not be avoided during the colourimetric assay.

Arithmetic mean of substrate concentration  $[\bar{S}]$  is an excellent approximation of true substrate concentration when substantial fraction of the substrate is utilized during the assay. If  $[S_0]$  is the initial substrate concentration and  $[S]$  is the substrate remaining at the end of the assay, then the modified Lineweaver-Burk (1934) equation assumes the form,

$$\frac{1}{\bar{v}} = \frac{K_m}{V_m [\bar{S}]} + \frac{1}{V_m}$$

Where,

$$\bar{v} = \frac{[S_0] - [S]}{t} \text{ and } [\bar{S}] = \frac{1}{2}([S_0] + [S]).$$

This procedure provided a more reliable estimate of  $K_m$  values. If the reaction goes to 50% completion, the  $K_m$  value estimated by this method will be high only by 4%. The results of the modified Lineweaver-Burk plots were analyzed by least-square curve fitting procedure (linear regression) using a programmable pocket calculator (Texas Instruments SR-51A).

<sup>b</sup> Enzyme could not be saturated due to limited solubility of Gln in water.

reaction velocity at a constant concentration of Gln (112.5 mM), NH<sub>2</sub>OH (50 mM) and ADP (0.5 mM) was highest around 0.35 mM Mn<sup>2+</sup> and from the sigmoid saturation pattern a  $K_{0.5}$  value of 70  $\mu$ M was calculated for Mn<sup>2+</sup> (table 4).

Figure 6 depicts the effect of pH on the Glu saturation of the Mg<sup>2+</sup>-supported synthetase activity of the enzyme. It can be seen from the linear Lineweaver-Burk plots that the slopes ( $K_m/V_m$ ) of the lines increase with decreasing pH. The inset of figure 6 shows a replot of  $-\log K_m$  versus pH. On drawing a tangent with an unit slope to this curve, a group with a  $pK_a$  value of 6.5 was implicated. This  $pK_a$  value approximated well with the value reported in the literature for a histidine in proteins.

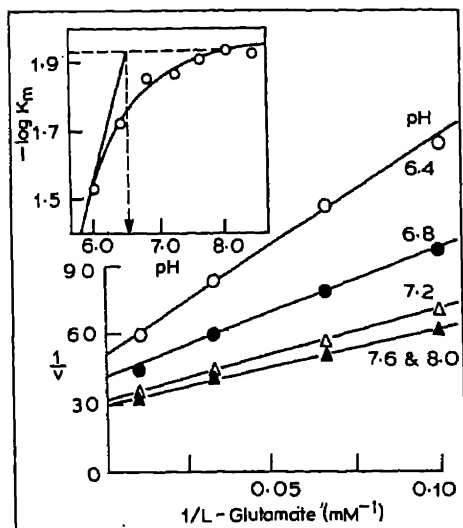


Figure 6. pH Dependence of saturation of the enzyme by Glu.

The  $Mg^{2+}$ -dependent synthetase activity was assayed in 100 mM imidazole, HCl buffer, pH 6.0, 6.4, 6.8, 7.2, 7.6, 8.0 or 8.4, at varying concentrations of Glu (0–150 mM) and at a fixed saturating concentration of  $NH_2OH$  (50 mM) and  $Mg$ . ATP (20:10 mM). From the Lineweaver-Burk plots, (all the curves are not given to avoid overcrowding but data were used in the calculation of  $pK_m$ ), the  $K_m$  values for Glu were determined at each pH value, by a least-square fit of the data. Inset:  $\log K_m$ , obtained at each pH value was plotted against corresponding pH. A  $pK_m$  value of 6.5 was calculated.

#### Functional amino acid residues at the active site

*A. niger* glutamine synthetase was rapidly inactivated by phenylglyoxal and increasing concentrations of the reagent enhanced the rate of inactivation. Figure 7 depicts the first-order plots obtained from the time course of inactivation at different concentrations of phenylglyoxal. In order to avoid overcrowding of the data, only a few representative curves are shown. A plot of  $\ln k_{app}$  (apparent first-order rate constant) as a function of  $\ln [\text{phenylglyoxal}]$  gave a straight line with a slope of 1.6 and from the intercept on the Y-axis, a second-order rate constant of  $3.8 \text{ M}^{-1} \text{ min}^{-1}$  was obtained (figure 7, Inset). When glutamine synthetase was incubated with 10 mM phenylglyoxal, it was rapidly inactivated (figure 8A and B). It can be seen from figure 8A that Glu,  $Mg^{2+}$  or  $NH_4^+$  had a marginal protective effect on the rate of inactivation whereas both ATP plus  $MgCl_2$  and  $Mn^{2+}$  afforded considerable protection against phenylglyoxal

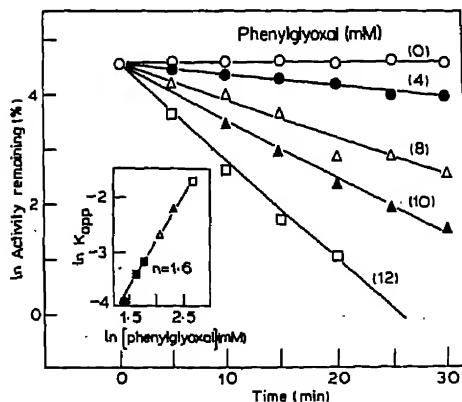
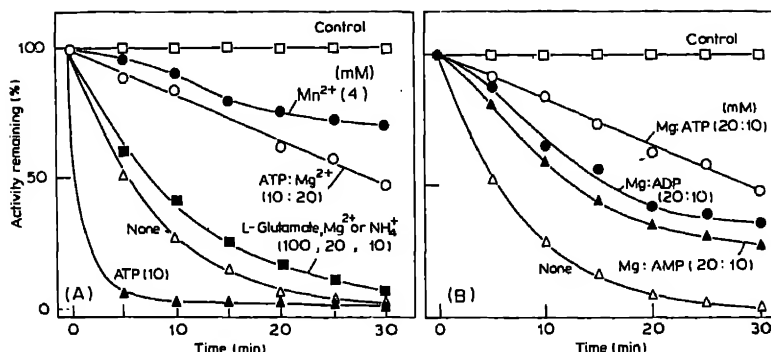


Figure 7. First order plots for the inactivation of the enzyme by phenylglyoxal (O, control; ●, 4 mM; ■, 5 and 6 mM; △, 8 mM; ▲, 10 mM; and □, 12 mM). Inset: The pseudo first order rate constant ( $k_{app}$ ) at each phenylglyoxal concentration was calculated from the slopes of the first order plots and  $\ln k_{app}$  is plotted against  $\ln$  phenylglyoxal concentration. A second order rate constant of  $3.8 \text{ M}^{-1} \text{ min}^{-1}$  was calculated.





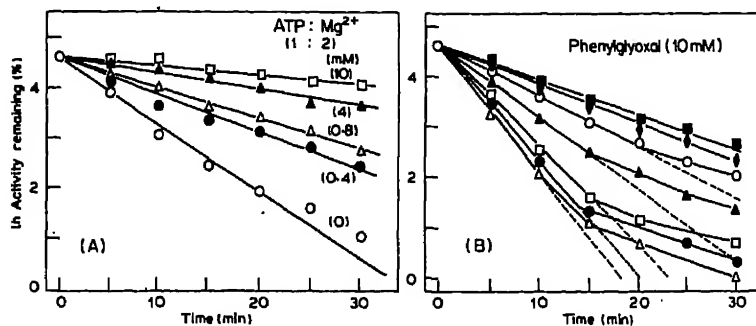
**Figure 8.** Protection of the enzyme by substrates and adenine nucleotides against phenylglyoxal (10 mM) inactivation.

**A.** The inactivation mixture in addition to the normal components, (please see materials and methods) contained: no addition ( $\Delta$ );  $Mn^{2+}$  ( $\bullet$ , 4 mM); ATP:  $Mg^{2+}$  ( $\circ$ , 10:20 mM); L-glutamate,  $Mg^{2+}$  or  $NH_4^+$  ( $\blacksquare$ , 100, 20, 10 mM, respectively); ATP ( $\blacktriangle$ , 10 mM).

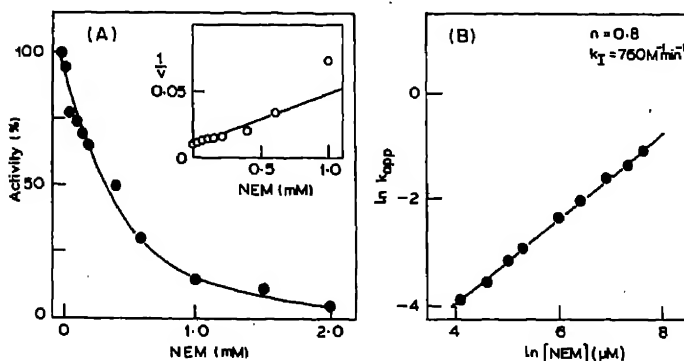
**B.** The inactivation mixture in addition to the normal components contained: no addition ( $\Delta$ ); ATP:  $Mg^{2+}$  ( $\circ$ , 10:20 mM); ADP:  $Mg^{2+}$  ( $\bullet$ , 10:20 mM); and AMP:  $Mg^{2+}$  ( $\blacktriangle$ , 10:20 mM). The control curve ( $\square$ ) in both A and B panels shows the absence of inactivation when no phenylglyoxal was added.

inactivation. The presence of free ATP greatly enhanced the inactivation rate. Similarly it is evident from figure 8B that ADP plus  $MgCl_2$  and AMP plus  $MgCl_2$  also protected the enzyme against inactivation, although the protection was a little less than that afforded by ATP plus  $MgCl_2$ . The order of effectiveness of protection by these three ligands was as follows:  $Mg \cdot ATP > Mg \cdot ADP > Mg \cdot AMP$ . The  $Mg \cdot ATP$  complex protected the enzyme against phenylglyoxal inactivation in a concentration dependent manner as shown by the first-order plots in figure 9A. Similarly, the protection afforded by increasing concentrations of  $Mn^{2+}$  (0–100  $\mu M$ ) against phenylglyoxal (10 mM) modification is depicted in figure 9B. From the concentration dependence of protection (figure 9), dissociation constant for the protective ligand was obtained using replots (Nelson *et al.*, 1962). These replots (not shown) gave the dissociation constants of 0.9 mM and 52  $\mu M$  for  $Mg \cdot ATP$  and  $Mn^{2+}$ , respectively.

When the enzyme was incubated for 10 min with increasing concentrations of NEM, enhanced loss of activity was observed (figure 10A). A replot of reciprocal of residual activity versus NEM concentration gave a straight line (figure 10A, Inset) indicating that the inactivation followed a first order kinetics and that the activity depended on a single/similar class of site(s) which were susceptible for NEM modification. In a separate experiment, time course of inactivation by increasing concentrations of NEM (0–2 mM) was monitored. From the first order plots (data not shown),  $k_{app}$  values were determined and a plot of  $\ln k_{app}$  versus  $\ln [NEM]$  was made. This plot gave a slope of close to one (0.8) and a second order rate constant of 760  $M^{-1} min^{-1}$  (figure 10B) was calculated. Like in the case of phenylglyoxal, Glu,  $NH_4^+$  or  $Mg^{2+}$  did not afford appreciable protection whereas  $Mn^{2+}$  and  $Mg \cdot ATP$  complex protected the enzyme considerably against the NEM modification (figure not given). The concentration dependence of protection against NEM inactivation by  $Mn^{2+}$  is shown in figure 11. By



**Figure 9.** A. First order plots for the protection afforded by ATP: Mg<sup>2+</sup> (1:2 ratio; O, 0 mM; ●, 0.4 mM; △, 0.8 mM; ▲, 4 mM; and □, 10 mM) against inactivation of the enzyme by phenylglyoxal (10 mM). B. First order plots for the protection afforded by Mn<sup>2+</sup> (△, 0 μM; ●, 5 μM; □, 10 μM; ▲, 20 μM; O, 50 μM; ◆, 70 μM; and ■, 100 μM) against inactivation of the enzyme by phenylglyoxal (10 mM).



**Figure 10.** A. Inactivation of the enzyme by increasing concentrations of NEM. Inset: Plot of  $1/v$  against NEM concentration.

B. Replot for the determination of second order rate constant and 'n' value for NEM inactivation.

The time course of the inactivation was carried out at different concentrations of NEM as described for phenylglyoxal (figure 7) and from the first order plots, the  $k_{app}$  values were calculated at each concentration of NEM. A second order rate constant of  $760 \text{ M}^{-1} \text{ min}^{-1}$  was obtained from the replot. Enzyme without 2-mercaptoethanol was used in all NEM inactivation studies.

suitably replotting this data (as mentioned above) a dissociation constant for Mn<sup>2+</sup>, of  $14 \mu\text{M}$  was calculated.

The pseudo first order rate constants ( $k_{app}$ ) for inactivation by both NEM (0.4 mM) as well as phenylglyoxal (10 mM) were determined in the presence of Mn<sup>2+</sup>, free ATP or different ratios of ATP: Mg<sup>2+</sup> and are summarized in figure 12. The effect of inclusion of various ligands was remarkably similar on the inactivation rate constants for NEM as well as phenylglyoxal. Whereas Mg . ATP complex and Mn<sup>2+</sup> protected

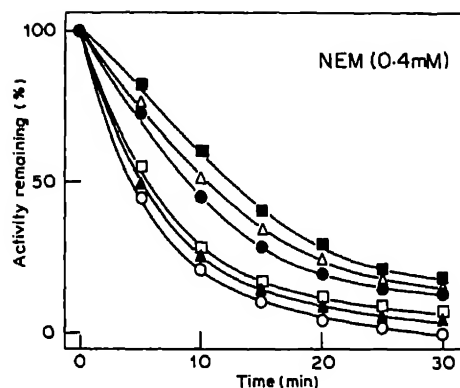


Figure 11. Protection by different concentrations of  $Mn^{2+}$  (O, 0  $\mu M$ ;  $\Delta$ , 3  $\mu M$ ;  $\square$ , 5  $\mu M$ ;  $\bullet$ , 50  $\mu M$ ;  $\Delta$ , 70  $\mu M$  and  $\blacksquare$ , 100  $\mu M$ ) against inactivation of the enzyme by NEM (0.4 mM).

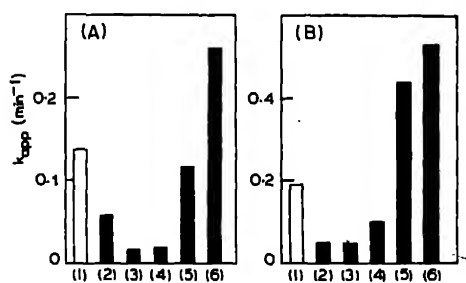


Figure 12. Summary of the pseudo first order rate constants for the inactivation of the enzyme. A. NEM (0.4 mM). B. Phenylglyoxal (10 mM) (1) Control, (2) +0.1 mM  $Mn^{2+}$ , (3) +10:20 mM ATP: $Mg^{2+}$ , (4) +10:10 mM ATP: $Mg^{2+}$  (5) +10:5 mM ATP: $Mg^{2+}$  and (6) +10 mM ATP.

the enzyme against inactivation (decreased  $k_{app}$  values), free ATP enhanced the rate of inactivation (increased  $k_{app}$  values) in both the cases.

## Discussion

The regulation of glutamine synthetase levels in *A. niger* appeared to be different from that observed in other fungi. In the case of *A. nidulans*, *Neurospora crassa* and *Candida utilis*, only Glu specifically elevated the levels of the enzyme, whereas in *A. niger* the enzyme levels were also enhanced by the presence of  $NO_3^-$  as the sole nitrogen source. One probable explanation for this observation could be the slow conversion of  $NO_3^-$  to  $NH_4^+$  (Pateman *et al.*, 1967) and as the  $NH_4^+$  concentrations did not reach high levels, glutamine synthetase activity was probably not repressed under these conditions. Repression of this enzyme levels by  $NH_4^+$  was indicated by the results presented in table 2.

The near basal levels of glutamine synthetase observed when *A. niger* was grown in a medium containing low concentrations of an easily assimilable nitrogen source, such as Gln or  $NH_4^+$  in addition to Glu or  $NO_3^-$  (table 1) suggested that Glu and  $NO_3^-$  are not specific inducers of this enzyme. A rational explanation for these observations is that the levels of glutamine synthetase respond reciprocally to the amount of easily

assimilable nitrogen supply. The fact that at low concentrations of  $\text{NH}_4^+$ , the enzyme levels are high (table 2), substantiate this suggestion. Further evidence in support of this contention was the observation that methylamine, which is a poor nitrogen source, enhanced the enzyme levels (table 1). The intensity of the protein stain (figure 2A) as well as the peak heights in the gel filtration profiles (figure 2) and the increased specific activity of glutamine synthetase present in the extracts, suggested that there was a larger amount of enzyme protein in the  $\text{Glu}$  and  $\text{NO}_3^-$  grown cells, compared to  $\text{NH}_4^+$  grown cells. These results are fully compatible with the above hypothesis.

In addition to regulation by  $\text{NH}_4^+$  repression, bacterial glutamine synthetase is also known to be modulated by covalent modification (Stadtman and Ginsburg, 1974) and the fungal enzyme by association-dissociation depending on the nitrogen status (Vichido et al., 1978; Sims et al., 1974b), as well as, by specific rapid inactivation (Ferguson and Sims, 1974b; Legrain et al., 1982; Van Andel and Brown, 1977). These mechanisms do not appear to be operating in the case of *A. niger* glutamine synthetase. The absence of regulation by covalent modification was indicated by the constant ratio of  $\text{Mg}^{2+}$ -dependent synthetase to  $\text{Mn}^{2+}$ -dependent  $\gamma$ -glutamyl transferase activity, a criterion employed to indicate this type of regulation (Pateman, 1969; Sims et al., 1974b). However, the identical elution profiles (figure 2) and electrophoretic mobility (figure 2A) under different growth conditions clearly indicated the absence of association-dissociation as a method of regulation of the enzyme in *A. niger*. Yeast glutamine synthetase was rapidly inactivated when a preferred nitrogen source e.g.,  $\text{NH}_4^+$  or glutamine was added to the exponentially growing cells (Ferguson and Sims, 1974b; Legrain et al., 1982; Van Andel and Brown, 1977). Such a mechanism may not be operating in *A. niger*, as  $\text{NH}_4^+$  and glutamine caused only a slow and marginal decrease in the activity (figure 3). The absence of rapid inactivating system in this fungus was also borne out by the fact that glutamine synthetase was not inactivated on mixing the crude extracts obtained from cells grown on different nitrogen sources (data not given). All these observations point to the differences in the regulatory mechanisms that operate in the control of this enzyme activity in different fungi.

Sporulation in *A. niger* was considerably delayed when grown on 100 mM  $\text{NO}_3^-$  (120 h compared to 72 h, figure 1). Rapid loss of glutamine synthetase activity just prior to sporulation suggested that, glutamine synthetase and hence the nitrogen status (Galbraith and Smith, 1969) could be a signal for sporulation in this fungus. Such a correlation between sporulation and glutamine metabolism has been observed in the case of *Bacillus subtilis* (Bott et al., 1977).

To further our studies on the molecular mechanisms of the regulation of glutamine synthetase, the enzyme from *A. niger* was purified to apparent homogeneity. The *A. niger* enzyme did not bind to many affinity matrices used in the purification of this enzyme (Seethalakshmi and Appaji Rao, 1979; Iyer et al., 1981; Lepo et al., 1979; Mitchell and Magasanik, 1983; Tuli and Thomas, 1981; Palacios, 1976). The enzyme was weakly adsorbed on to Glu-Sepharose (Lin and Kapoor, 1978), probably by ionic interactions rather than by specific bioaffinity. These observations again highlight the differences in the properties of *A. niger* glutamine synthetase compared to the enzyme from other sources.

The  $M_r$  of the native glutamine synthetase from *A. niger* ( $385,000 \pm 25,000$ ) was similar to the enzyme reported from other fungal sources (Sims et al., 1974a; Palacios,

1976). From the SDS-polyacrylamide gel electrophoresis of the enzyme it was established that the *A. niger* enzyme was octameric and was composed of identical subunits. In this respect, glutamine synthetase from *A. niger* resembles other fungal and mammalian enzymes (Stahl and Jaenicke, 1972; Tate and Meister, 1973). Glycerol (5%) effectively stabilized the enzyme against inactivation during storage and also against repeated freezing and thawing.

The characteristic influence of the metal ion activators on the pH optimum of the enzyme (figure 5) may have some physiological significance for this organism as it excretes citric acid under acidic pH conditions and  $Mn^{2+}$  deficiency (Kubicek and Rohr, 1978). Intracellular pH and the ratio of  $Mg^{2+}$  to  $Mn^{2+}$  inside the cell could be regulating the glutamine synthetase activity.

Like glutamine synthetase from all the other sources, *A. niger* enzyme also exhibited hyperbolic saturation patterns with Glu, Mg.ATP and  $NH_2OH$  (or  $NH_4^+$ ). A plot of  $pK_m$  against pH, indicated that the binding of Glu to the enzyme required an ionizable group with a  $pK_a$  of 6.5 probably a histidine (figure 6, Inset). Such a pH dependent Glu saturation was reported for mammalian glutamine synthetase (Schnackerz and Jaenicke, 1966). None of the ionizable groups of the substrates titrate with a  $pK_a$  value of 6.5. The high  $K_m$  value for Gln (235 mM), may account for the rapid breakdown of [E. glutamine] complex thereby making the biosynthetic reaction of *A. niger* glutamine synthetase essentially irreversible.

The availability of highly purified *A. niger* glutamine synthetase in reasonable amounts, permitted a critical evaluation of the essential amino acid residues required for activity. Arginine and cysteine have been implicated as active site amino acids in glutamine synthetases (Shapiro and Stadtman, 1967; Uralets *et al.*, 1977; Rao *et al.*, 1973; Jaenicke and Berson, 1977; Powers and Riordan, 1975). Phenylglyoxal was used to locate reactive arginine residues in the *A. niger* glutamine synthetase. From the inactivation data, replot of the pseudo first order plots (figure 7) suggested that an arginine residue on the enzyme was probably reacting with 2 mol of phenylglyoxal. Such a reaction has been observed with active site arginine residues in several enzymes (Riordan, 1979). Of the three substrates, only Mg.ATP complex protected the enzyme against inactivation, in a concentration dependent manner and the constant for protection calculated (0.9 mM, figure 9A) was comparable to the  $K_m$  value of Mg.ATP complex (1.5 mM, table 4). These results would suggest that the reactive arginine residue may be the Mg.ATP binding subsite at the active site. This was further substantiated by the fact that Mg.ADP and Mg.AMP which are competitive inhibitors of the enzyme with respect to Mg.ATP (data not shown), also protected it against phenylglyoxal inactivation. The observation that  $k_{app}$  values for the three ligand complexes followed the order, Mg.AMP > Mg.ADP > Mg.ATP, also point to the occurrence of arginine at the active site. If the interaction of the nucleotide with arginine involves the phosphate group(s) of the nucleotide, it is to be expected that the reduction in the number of phosphates would decrease the strength of interaction.

Almost all the glutamine synthetases studied so far are thiol proteins, as shown by the decrease of enzyme activity by a variety of thiol inhibitors *e.g.*, *p*-hydroxymercuribenzoate (pHMB), NEM, iodoacetate and 5,5-dithio-*bis*-2-nitrobenzoic acid. The results using NEM (figure 10A and B) clearly point out that a reactive cysteine may be essential for the activity of *A. niger* glutamine synthetase.

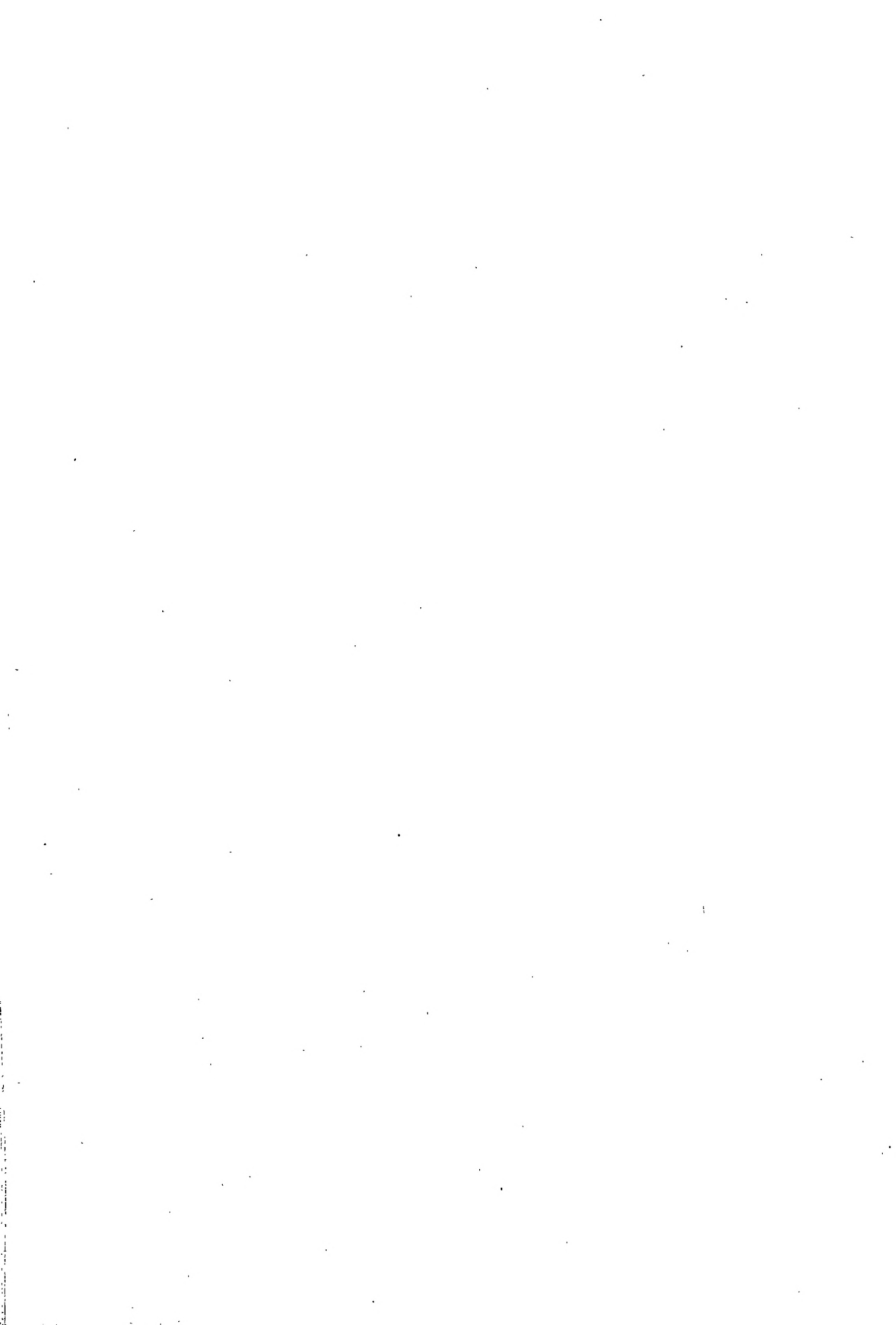
Low concentrations of manganese protected the enzyme against inactivation by both phenylglyoxal and NEM (figures 9B and 11). Based on the concentration dependence of this protection, the dissociation constants of 52  $\mu\text{M}$  and 14  $\mu\text{M}$  for  $\text{Mn}^{2+}$  were calculated (which are in reasonably close agreement, taking into account the limitations of the methods used). This data also suggested the presence of a single high affinity  $\text{Mn}^{2+}$  binding site on the enzyme. The kinetic constant for  $\text{Mn}^{2+}$  obtained from steady state kinetic analysis (Punekar, 1983) was atleast an order of magnitude higher than the binding constants obtained from protection experiments, indicating the presence of more than one class of  $\text{Mn}^{2+}$  binding sites on the enzyme. In the case of *E. coli* glutamine synthetase, based on the protection experiments, it was demonstrated that  $\text{Mn}^{2+}$  binding at the high affinity site protected the enzyme against inactivation reflecting the stability of the 'taut' form of the enzyme (Shapiro and Stadtman, 1967). It is evident that like in the glutamine synthetases from other sources (Hunt and Ginsburg, 1980; Shapiro and Stadtman, 1967; Uralets et al., 1977; Jaenicke and Berson, 1977). *A. niger* enzyme also probably requires a histidine, arginine and a sulphhydryl group for catalytic activity.

Although *A. niger* enzyme is regulated differently from the enzyme from other bacterial and fungal sources, the active site residues seem to be well conserved.

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## Lipoic acid and diabetes: Effect of dihydrolipoic acid administration in diabetic rats and rabbits

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**Abstract.** Relative  $\alpha$ -lipoic acid content of diabetic livers was considerably less than that of normal livers as determined by gas chromatography. It was not possible to detect any dihydrolipoic acid in the livers. Biochemical abnormalities such as hyperglycaemia, ketonemia, reduction in liver glycogen and impaired incorporation of  $[2-^{14}\text{C}]$ -acetate into fatty acids in alloxan diabetic rats were brought to near normal levels by the oral or intraperitoneal administration of dihydrolipoic acid. The effect of  $\alpha$ -lipoic acid was comparable to that of dihydrolipoic acid in reducing the blood sugar levels of diabetic rabbits during a glucose tolerance test.

The results suggest that the mode of action of lipoic acid was through stimulation of pyruvate dehydrogenase.

**Keywords.**  $\alpha$ -Lipoic acid; dihydrolipoic acid; gas chromatography; alloxan diabetic rats; blood glucose; acetoacetate; liver glycogen; fat synthesis.

### Introduction

$\alpha$ -Lipoic acid is a vital cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of  $\alpha$ -keto acids (*e.g.*, pyruvate,  $\alpha$ -ketoglutarate and branched chain  $\alpha$ -keto acids). There is an impairment of pyruvate oxidation in diabetes as evidenced by the decreased levels of the active form of pyruvate dehydrogenase (EC 2.4.1.1) (Kerbey *et al.*, 1976). Consequently, a large proportion of the acetyl coenzyme A (CoA) needed for energy generation is obtained through fatty acid oxidation resulting in an increased ratio of acetyl CoA/CoA in the mitochondria and increased production of ketone bodies (Ontko, 1972; Kerbey *et al.*, 1976; Randle *et al.*, 1977). Further, the increased gluconeogenesis in diabetes has been shown to be due to enhanced activities of several cytosolic enzymes such as phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-bisphosphatase (EC 3.1.3.11) and the mitochondrial enzyme pyruvate carboxylase (EC 6.4.1.1) (Prinz and Seubert, 1964; Wilmhurst and Manchester, 1970). In streptozotocin induced diabetes, pyruvate carboxylase activity is significantly increased whereas a larger proportion of pyruvate dehydrogenase is in its inactive state (Weinberg and Utter, 1980). Thus, it becomes apparent that the metabolism of pyruvate is a crucial regulatory step in diabetes because it is a metabolite located at the crossroads of the three major pathways impaired in diabetes, *i.e.*, glycolysis, gluconeogenesis and

Abbreviations used: DTO, Dihydrolipoic acid; LS<sub>2</sub>, lipoic acid; CoA, coenzyme A; NMR, nuclear magnetic resonance.

amino acid biosynthesis. Further, Wada *et al.* (1960) have reported that the levels of  $\alpha$ -lipoic acid in the blood and liver in alloxan diabetic rats are considerably reduced. In view of this, it was considered useful to evaluate the role of  $\alpha$ -lipoic acid in diabetes.

$\alpha$ -Lipoic acid has been estimated by several procedures including microbiological methods (Wada *et al.*, 1960; Herbert and Guest, 1975), gas chromatographic (Shih and Steinsberger, 1981; White, 1981) and high performance liquid chromatography (Howard and McCormick, 1981). The microbiological assay does not distinguish between  $\alpha$ -lipoic and dihydrolipoic acid and further the assay does not work in the presence of excess glucose. Hence, this procedure can be expected to give erroneous results in diabetes because of the rather high glucose concentrations. In the present study we have estimated the levels of  $\alpha$ -lipoic and dihydrolipoic acid in the livers of normal and alloxan diabetic rats using a gas chromatographic procedure. The effect of  $\alpha$ -lipoic and dihydrolipoic acid administration on some of the major biochemical abnormalities in diabetic rats has also been investigated.

In this context, it may be mentioned that several investigators have attempted to reduce blood sugar in human diabetes by administration of  $\alpha$ -lipoic acid (Pagliaro, 1956; Pagliaro and Furitano, 1956; Greco, 1957; Brusa and Serafini, 1958; Zueva, 1970). No efforts have been made to elucidate the possible mode of action of lipoic acid. In the present study, it was found that  $\alpha$ -lipoic acid was considerably reduced in the liver of diabetic rats and administration of  $\alpha$ -lipoic and dihydrolipoic acid had a beneficial effect on all the biochemical aberrations measured in diabetic rats.

## Materials and methods

DL  $\alpha$ -lipoic acid was purchased from British Drug House Ltd., Poole, England. Alloxan monohydrate was obtained from SD Fine Chemicals, Bombay. [ $2\text{-}^{14}\text{C}$ ]-Acetate was procured from Bhabha Atomic Research Centre, Bombay, and all other reagents were of analytical grade. Male rats and rabbits used in this study were an inbred Haffkine strain.

### Analytical methods

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WH 90 instrument operating under ASPECT 2000 control at 90 and 23 MHz for  $^1\text{H}$  and  $^{13}\text{C}$  respectively using  $\text{CDCl}_3$  as solvent.

For gas chromatographic analysis, methylation was carried out by reacting lipoic acid (106 mg, 0.5 mmol) or dihydrolipoic acid (106 mg, 0.5 mmol) with anhydrous methanolic  $\text{HCl}$  (5 %, 2 ml) at  $60^\circ\text{C}$  for 20 min. After reaction, the mixture was flushed with  $\text{N}_2$  gas to dryness. The methyl ester was redissolved in  $\text{CHCl}_3$  for analysis.

Methylated compounds were analysed on a Hewlett Packard 5730 A gas chromatograph. The column was 3 % SP-1000 coated on Supelcoport (100–120).  $\text{N}_2$  was the carrier gas and the compounds were detected by flame ionization. The column temperature was programmed from  $150^\circ\text{C}$ – $240^\circ\text{C}$  at a rate of  $2^\circ\text{C}/\text{min}$ . The temperature of the detector and injector were  $300^\circ\text{C}$  and  $250^\circ\text{C}$  respectively.

Radioactivity measurements were performed on a Packard Tricarb liquid scintillation spectrometer. Blood and urinary sugar were determined according to the method of Somogyi (1952).

#### *Preparation and estimation of dihydrolipoic acid*

The procedure used by Hager and Gunsalus (1953) was modified as follows: DL  $\alpha$ -lipoic acid (212 mg, 1 mmol) was dissolved in 0.25 M potassium bicarbonate (12 ml). Sodium borohydride (48 mg, 1.5 mmol) was added to the stirred solution keeping the temperature below 5°C. The reaction mixture was acidified to pH 2.0 with chilled 5 N HCl and extracted with benzene. The organic layer was washed with water, dried over anhydrous sodium sulphate and the solvent distilled under reduced pressure.

The sulphydryl content of dihydrolipoic acid was estimated using the procedure of Alexander (1958).

#### *Isolation of lipoic acid from rat liver*

Rats of two age groups (25 and 45 days old) were used in this study. Some rats in each age group were made diabetic by subcutaneous injection of alloxan (180 mg/kg) in acetate buffer (0.1 M, pH 5.4). Rats excreting more than 10 g/l sugar in urine were used in the study.

$\alpha$ -Lipoic acid was isolated from the livers of rats as described by Carreau (1979). Normal and diabetic rats (4 each in the two age groups) were sacrificed, the livers removed, washed with saline, blotted dry and weighed. Wet liver (1 g) was homogenized in Potter-Elvehjem homogenizer with 7 ml of water at 4°C. Homogenization was continued first with the addition of 17 ml methanol followed by 17 ml chloroform and finally 8 ml of water. The resulting mixture was centrifuged at 2000 *g* at 0°C for 20 min. Three layers separated: (i) upper aqueous methanolic layer, (ii) interphase and (iii) lower chloroform layer. The layers were separated, the interphase was homogenized with a 2:1 mixture of water and chloroform and centrifuged as before. The aqueous and chloroform layers were separated and mixed with the corresponding layers from the first centrifugation. This operation was repeated four more times.

The pooled aqueous methanolic extracts were concentrated on a rotary evaporator and the residue was dissolved in 0.5 ml of 6 N HCl and hydrolyzed in a sealed tube at 110°C for 16 h. After evaporating the HCl, the residue was extracted with chloroform. The chloroform layer was evaporated with a stream of N<sub>2</sub> gas and the lipoic acids were separated on thin layer chromatography with chloroform:methanol:water:formic acid (75:25:4:1; v/v). Pure lipoic and dihydrolipoic acid (visualised on a separate plate with a solution of 0.5 g of palladium chloride, 100 ml water, 400 ml ethanol and 10 drops of conc. HCl) regions were cut out, extracted with chloroform:methanol (1:1, v/v). The extract was filtered, the solvent evaporated and the residue esterified with methanolic HCl.

The methylated compounds were filtered through a microcolumn (0.5 × 3 cm) of silicic acid (100 mesh) and the samples used for gas chromatography.

*Glucose tolerance test in normal and diabetic rabbits*

Four normal and six alloxan diabetic rabbits weighing about 2.5 kg were starved for 18 h and their fasting blood sugar determined. Dihydrolipoic acid or  $\alpha$ -lipoic acid ( $100 \mu\text{mol/kg}$ ) was injected intraperitoneally as a suspension in 0.5 ml of 0.8% saline in two normal and three alloxan diabetic rabbits. The remaining animals received 0.5 ml of saline alone. Two h later a 40% solution of glucose in water ( $2\text{g/kg}$ ) was administered orally. Blood sugar (ear vein) was estimated at hourly intervals upto 4 h.

*Blood sugar in diabetic rats*

Nine alloxan diabetic rats weighing about 100 g were divided into two groups. Dihydrolipoic acid ( $500 \mu\text{mol/kg}$ ) was administered orally as a suspension in saline in six animals and three animals receiving saline alone served as controls. Blood was drawn by cardiac puncture at 0, 2 and 4 h following dihydrolipoic acid administration and the sugar content determined.

*Acetoacetate levels in diabetic rats*

Dihydrolipoic acid ( $500 \mu\text{mol/kg}$ ) was administered as a suspension in saline to four alloxan diabetic rats. Blood was collected by cardiac puncture before and 24 h after dihydrolipoic acid administration and acetoacetate was estimated by the method of Walker (1954).

*Liver glycogen in normal and diabetic rats*

Six normal and 6 alloxan diabetic rats weighing about 100 g were distributed into two groups. Dihydrolipoic acid ( $500 \mu\text{mol/kg}$ ) as a suspension in saline was injected intraperitoneally to three normal and three diabetic rats. The remaining animals were injected saline alone. The animals were sacrificed 24 h later, the livers removed, washed with a solution of saline, blotted dry and weighed. The glycogen content was determined according to the method of Hassid and Abraham (1956).

*[2- $^{14}\text{C}$ ]-Acetate incorporation into liver lipids in normal and diabetic rats*

Dihydrolipoic acid ( $500 \mu\text{mol/kg}$ ) was injected intraperitoneally into three normal and four diabetic rats. Three normal and 4 diabetic rats administered saline only served as controls. Two h later [2- $^{14}\text{C}$ ]-acetate ( $4 \mu\text{Ci}/100\text{g}$ ) was injected in all the animals. Twenty four h after dihydrolipoic acid administration, the animals were sacrificed, the livers removed, washed with a solution of saline, blotted dry and weighed. The wet livers were homogenized in chloroform:methanol (2:1 v/v) in an Omnimixer for 5 min at 3000 rpm. The organic layer was separated by filtration, washed with a 0.03% magnesium chloride solution and then with water. The solvent was distilled under reduced pressure and the residue (lipids) was weighed to a constant weight. The total radioactivity in the residue was determined.

## Results

### *GC analysis of methyl lipoate and methyl dihydrolipoate*

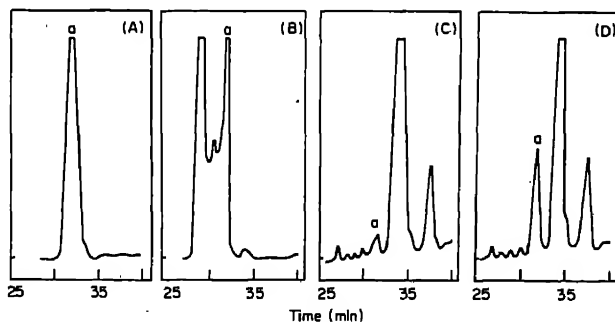
Methyl lipoate gave a single peak with a retention time of 32.9 min (figure 1, panel A). However, methyl dihydrolipoate gave two peaks with retention times of 28.4 and 32.9 min (figure 1, panel B). The relative intensities of these two peaks were 40:56. The latter corresponds to methyl lipoate and the former presumably to methyl dihydrolipoate. It was therefore necessary to establish whether the methyl dihydrolipoate was contaminated with methyl lipoate or whether during GC analysis it was getting oxidized to methyl lipoate. Purity analysis was carried out using  $^{13}\text{C}$  NMR. From this analysis it was concluded that methyl dihydrolipoate was 95% pure. Therefore, it appears that during GC analysis 56% of the methyl dihydrolipoate gets oxidized. As long as the extent of this reaction is taken into account, GC analysis of liver samples can be used for quantitative estimation of dihydrolipoic acid levels.

### *Levels of $\alpha$ -lipoic and dihydrolipoic acid in rat livers*

The presence of  $\alpha$ -lipoic acid was detected in all the liver samples tested by this method. Despite the presence of unidentified peaks in the gas chromatogram,  $\alpha$ -lipoic acid could be identified as a distinct peak with a retention time of 32 min (figure 1, panel C). Inclusion of a known quantity of methyl lipoate as an external standard showed that the intensity of the peak with a retention time of 32 min was increased by the corresponding amount (figure 1, panel D). The relative peak areas before and after addition of standard methyl lipoate was a measure of the lipoic acid content in the sample (table 1). In alloxan diabetic rat livers the  $\alpha$ -lipoic acid content was one fourth of that found in normal livers.

Dihydrolipoic acid could not, however, be detected in any of the samples analysed. Taking into account the sensitivity of this method, it was estimated that the levels of dihydrolipoic acid, if present, must be less than 0.5  $\mu\text{g/g}$  tissue.

The results of the glucose tolerance test are shown in table 2. Dihydrolipoic acid and



**Figure 1.** Gas chromatography analysis of methyl lipoate and methyl dihydrolipoate. Panel A, Pure methyl lipoate (a). Panel B, Methyl dihydrolipoate. Panel C, Liver extract. Panel D, Same extract with externally added methyl lipoate.

**Table 1.**  $\alpha$ -Lipoic acid content of livers from normal and alloxan diabetic rats.

Age (days)	$\alpha$ -Lipoic acid content ( $\mu\text{g/g}$ wet tissue)		
	Normal (N)	Diabetic (D)	Ratio (N/D)
25	13.1 $\pm$ 0.05	3.85 $\pm$ 1.6	3.4
45	8.0 $\pm$ 2.1	2.05 $\pm$ 0.6	3.9

All values are means of 4 rats  $\pm$  S.E.

**Table 2.** Glucose tolerance test in normal and diabetic rabbits.

	Blood glucose mg/100 ml (mean $\pm$ SE)				
	Hours				
	0	1	2	3	4
Normal (2)	72	152	105	87	90
Normal (2) + DTO	74	129	102	80	84
Diabetic (3)	257 $\pm$ 34	449 $\pm$ 62	494 $\pm$ 17	485 $\pm$ 37	446 $\pm$ 46
Diabetic (3) + DTO	245 $\pm$ 35	375 $\pm$ 34	319 $\pm$ 67	287 $\pm$ 12	276 $\pm$ 49
P value			< 0.1	< 0.05	< 0.05
Diabetic (3)	145 $\pm$ 15	261 $\pm$ 28	271 $\pm$ 27	248 $\pm$ 24	188 $\pm$ 6
Diabetic (3) + LS <sub>2</sub>	152 $\pm$ 12	179 $\pm$ 15	212 $\pm$ 15	178 $\pm$ 15	162 $\pm$ 22
P value		< 0.05		< 0.05	

Numbers in parenthesis indicate the number of animals.

$\alpha$ -lipoic acid administration in alloxan diabetic rabbits brought about a significant reduction in the blood sugar levels in a paired 't' test at all the time points tested. Furthermore, the profile of the glucose tolerance test resembled the normal profile more closely. Neither dihydrolipoic acid nor  $\alpha$ -lipoic acid had any significant effect on blood glucose levels in normal rabbits.

Similarly, it was found that dihydrolipoic acid administered orally also brings about a significant reduction in the blood sugar levels of diabetic rats (table 3). The mean reduction in the blood sugar levels at the end of 2 and 4 h was 22% and 38% respectively.

Acetoacetate levels in the blood were reduced by a greater than two fold margin following dihydrolipoic acid administration in diabetic rats (table 4).

In addition to bringing about a reduction in the blood sugar and acetoacetate levels in diabetic rats, dihydrolipoic acid increases the liver glycogen (table 5) and [2-<sup>14</sup>C]-acetate incorporation into liver lipids (table 6) by a greater than two fold margin. At the

**Table 3.** Blood glucose levels in diabetic rats.

	Blood glucose mg/100 ml (mean $\pm$ SE)		
	Hours		
	0	2	4
Diabetic (3)	345 $\pm$ 51	343 $\pm$ 40*	333 $\pm$ 22*
Diabetic + DTO (6)	421 $\pm$ 55	328 $\pm$ 51**	261 $\pm$ 38***

Numbers in parenthesis indicate the number of animals.

\* Not significantly different from 0 h reading.

\*\* Significantly different from 0 h reading at  $P < 0.1$ .

\*\*\* Significantly different from 0 h reading at  $P < 0.01$ .

**Table 4.** Acetoacetate levels in blood of diabetic rats.

	Acetoacetate (mg/100 ml)	<i>P</i> value
Normal	0.78 $\pm$ 0.1	
Diabetic	11.5 $\pm$ 1.0	< 0.001
Diabetic	11.5 $\pm$ 1.0	
Diabetic + DTO	4.1 $\pm$ 1.2	< 0.01

All values are means of 4 animals  $\pm$  SE.

**Table 5.** Liver glycogen in normal and diabetic rats.

	Liver glycogen g per cent	<i>P</i> value
Normal	3.29 $\pm$ 0.35	
Normal + DTO	2.89 $\pm$ 0.21	NS*
Diabetic	0.74 $\pm$ 0.06	
Diabetic + DTO	1.69 $\pm$ 0.27	< 0.01

All values are means of 3 animals  $\pm$  SE.

\* Not significant.

same time, these two constituents are not affected significantly in normal rats administered dihydrolipoic acid.

## Discussion

It is evident that  $\alpha$ -lipoic acid levels are significantly reduced in rat livers during alloxan diabetes. Wada *et al.* (1960) had noted a similar reduction in  $\alpha$ -lipoic acid in diabetic

Table 6. Incorporation of [2-<sup>14</sup>C]-acetate into liver lipids.

	CPM incorporated $\times 10^3$		P value
	Per g wet tissue	Per g of fat	
Normal (3)	5.9 $\pm$ 1.3	141.6 $\pm$ 21	NS*
Normal + DTO (3)	4.5 $\pm$ 0.4	109.6 $\pm$ 16	
Diabetic (4)	1.5 $\pm$ 0.3	43.5 $\pm$ 9.1	< 0.05
Diabetic + DTO (4)	3.7 $\pm$ 0.5	85.5 $\pm$ 5.3	

Numbers in parenthesis indicate the number of animals  $\pm$  SE.

\* Not significant.

rats. However, they had employed the microbiological assay which has several limitations as pointed out earlier. We have also shown that dihydrolipoic acid could not be detected in the livers of normal or alloxan diabetic rats. It is interesting to note that Shih and Steinsberger (1981) employing a similar extraction followed by GC analysis also failed to detect any dihydrolipoic acid in chick liver. It appears reasonable to conclude therefore, that  $\alpha$ -lipoic acid exists mainly as the disulphide and not the sulphydryl form in liver.

Lipoic acid exists predominantly in the protein bound form as the  $\epsilon$ -aminolysyl amide (Reed, 1966). The method of extraction employed in this study is designed to measure only protein bound lipoic acid. It is, of course, possible that some of the  $\alpha$ -lipoic acid may be degraded during acid hydrolysis. However, it is expected that this loss does not affect the ratio of  $\alpha$ -lipoic acid levels in normal and alloxan diabetic rat livers.

Results of  $\alpha$ -lipoic and dihydrolipoic acid administration show that the four major biochemical abnormalities in diabetes *viz.*, hyperglycaemia, ketonemia, reduced glycogen and fat synthesis, have been corrected to a significant extent by the administration of dihydrolipoic acid in diabetic rats. On the other hand, dihydrolipoic acid did not affect any of these parameters to a significant extent in normal animals. In addition, dihydrolipoic acid increases the tolerance to glucose in diabetic rabbits during a glucose tolerance test. In this system the effect of  $\alpha$ -lipoic acid is very similar to that of dihydrolipoic acid.

Diabetes is a disease characterised by an aberration of glucose metabolism and transport (Foster, 1978; Karnielli *et al.*, 1981). Insulin administration has a general beneficial effect on both these parameters. It is difficult to assess the relative contributions made by these two effects in diabetes because of the difficulty in studying them in isolation. However, it is safe to say that both effects are extremely important for the proper management of glucose levels in the blood. The mechanism of action of insulin in improving glucose metabolism has been ascribed generally to the stimulation of several enzymes involved in glycolysis, tricarboxylic acid cycle and fatty acid biosynthesis (Denton *et al.*, 1981). The pivotal enzyme at the crossroads of these three pathways is pyruvate dehydrogenase (EC 1.2.4.1) which is a multienzyme complex catalyzing the oxidative decarboxylation of pyruvate to acetyl CoA. In view of the



importance of this reaction in the metabolism of glucose, the role of insulin in regulating the activity of this enzyme is of considerable interest.

The predominant mode of regulation of pyruvate dehydrogenase has been shown to be by a phosphorylation (inactive enzyme) and dephosphorylation (active enzyme) mechanism. Insulin brings about a reversible dephosphorylation, thus converting the inactive to the active form (Hughes *et al.*, 1980). The importance of the activation of pyruvate dehydrogenase in mediating the hypoglycaemic effect of insulin is evidenced by the fact that dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase also brings about an activation of pyruvate dehydrogenase, and has been shown to induce hypoglycaemia in diabetic and starved normal rats (Whitehouse and Randle, 1973; Blackshear *et al.*, 1974). Thus, one may presume that at least a part of the hypoglycaemic effect of insulin is mediated by the activation of pyruvate dehydrogenase.

The question, therefore, is whether the beneficial effects of dihydrolipoic acid and  $\alpha$ -lipoic acid administration in diabetes is mediated through insulin secretion or directly through the activation of pyruvate dehydrogenase. Since alloxan administration results in a near total destruction of  $\beta$ -cells of the pancreas, neither  $\alpha$ -lipoic or dihydrolipoic acid release any extra insulin over and above that released by the high glucose levels present in diabetic rats (Malaisse *et al.*, 1982).

Evidence for the alternative hypothesis stems from the fact that the activity of pyruvate dehydrogenase is lowered in alloxan diabetes (Kerbey *et al.*, 1976). Administration of insulin restores only a part of the activity of this enzyme (Stansbie *et al.*, 1980; Wieland *et al.*, 1971). This points to an irreversible diminution of a part of this enzyme activity in alloxan diabetic rats, perhaps due to lower levels of  $\alpha$ -lipoic acid in the liver. It would be reasonable to conclude therefore, that administration of  $\alpha$ -lipoic acid or dihydrolipoic acid restores the activity of this enzyme thus alleviating some of the biochemical aberrations in diabetes. Definitive evidence indicating the enhancing effect of lipoic acid on liver pyruvate dehydrogenase will be presented in a subsequent communication.

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## Isolation and partial characterisation of $\alpha$ -amylase components evolved during early wheat germination

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**Abstract.** The development of  $\alpha$ -amylase (EC 3.2.1.1) activity in wheat was followed during 4 days of germination. The enzyme was purified and separated by gel chromatography into two distinct entities ( $\alpha$ -amylase I and  $\alpha$ -amylase II), with different molecular weights and isoelectric points.  $\alpha$ -Amylase I contained a much higher content of sugars than  $\alpha$ -amylase II, which decreased as the germination proceeded. The time sequence analysis of the starch degradation pattern showed that on the 4th day of germination, 15 % of the total activity was present in  $\alpha$ -amylase I and the rest in  $\alpha$ -amylase II. Similarly, differences in the relative rates of synthesis of their isoenzymes were observed.  $\alpha$ -Amylase I was resolved on the 4th day of germination, only into 3 isoenzymes, whereas  $\alpha$ -amylase II could separate into 4 isoenzymes. The enzyme activity was however maximal in the most electropositive isoenzyme in both the components.

**Keywords.** Wheat;  $\alpha$ -amylases I and II; germination; development; physicochemical characterization.

### Introduction

$\alpha$ -Amylase ( $\alpha \rightarrow 1$ -4-glucan 4-glucanhydrolase, EC 3.2.1.1) is a key enzyme hydrolysing reserve starch in the endosperm of germinating cereals. It is synthesized *de novo* in the aleurone cells, in response to gibberellic acid (Yomo and Varner, 1971). Scutellar epithelium has also been indicated as the dominant site of the enzyme formation in germinating rice seeds (Okamoto and Akazawa, 1979). Different  $\alpha$ -amylases are known to be evolved during germination (Frydenberg and Nielsen, 1965), and consist of number of isoenzymes (Jacobsen *et al.*, 1970). However, the mechanism by which the isoenzymes are transported from the site of synthesis to the endosperm is not well understood. It was suggested that the enzyme was released by simple diffusion (Varner and Mense, 1972) and a partial degradation of cell walls facilitated this process (Ashford and Jacobsen, 1974). A soluble mode of secretion (Jones, 1973) as well as through a secretory vesicles derived from endoplasmic reticulum (Locy and Kende, 1978) was reported. Recently, the transport of the membrane-bound enzyme across the membrane was explained by signal hypothesis (Boston *et al.*, 1980). Poly(A)-enriched

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Abbreviations used: BSA, Bovine serum albumin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

mRNA, which synthesizes polypeptide precursor of  $\alpha$ -amylase, was isolated from the aleurone layers of barley (Mozer, 1980) and wheat (Okita *et al.*, 1979). Cereal  $\alpha$ -amylases were shown to be glycoproteins (Rodaway, 1978; Miyata *et al.*, 1981) like other secretory proteins.

In the present investigation, the formation of two different entities of  $\alpha$ -amylase during wheat germination was monitored and their relative amounts estimated. The isoenzymes were partially characterized physico-chemically. Highly glycosylated  $\alpha$ -amylase I exhibited significantly low enzymic activity compared to the activity of  $\alpha$ -amylase II, with a low carbohydrate content.

## Materials and methods

### Seeds and chemicals

Newly harvested 'Vijay' variety of wheat samples were obtained from the Agricultural Research Station, Niphad, Maharashtra. The seeds had about 100% viability. The molecular weight standards [bovine serum albumin (BSA), ovalbumin, pepsin, chymotrypsin], polyvinyl pyrrolidone and sugar standards were from Sigma Chemicals, St. Louis, Missouri, USA. Other chemicals and solvents used were of Analar grade.

### Extraction of $\alpha$ -amylase

Wheat seeds (10 g lots) were washed with 1% NaOCl solution, rinsed and imbibed with distilled water for 16 h. Germination was carried out in the dark at 25°C for 2 to 4 days on filter paper moistened with streptomycin and penicillin (5  $\mu$ g/ml) to prevent microbial growth. The vegetative portions were discarded and the endosperm tissues were homogenised at 3°C in 0.01 M sodium acetate buffer (pH 5.6) containing 0.003 M  $\text{CaCl}_2$  and 1% polyvinyl pyrrolidone. The extract was centrifuged at 5,000 *g* for 10 min and the supernatant adjusted to pH 8.0 with 1 N NaOH. The solution was heated to 70°C to inactivate  $\beta$ -amylase, cooled (4°C) and centrifuged. The pellet was discarded and the supernatant fraction was used as the crude enzyme preparation.

### Enzyme purification

The crude extract was adjusted to 45%  $(\text{NH}_4)_2\text{SO}_4$  saturation, kept for 16 h at 4°C and centrifuged at 12,000 *g*. The precipitate was dissolved in 0.01 M acetate buffer (pH 5.6) containing 3 mM  $\text{CaCl}_2$  and dialysed against the same buffer for 6 h. The dialysed enzyme was chromatographed on a Sepharose 4B column (2.5  $\times$  15 cm), equilibrated with 0.05 M Tris-HCl buffer (pH 8.2) containing 0.05 M  $\text{Na}^+$  and 0.003 M  $\text{Ca}^{2+}$  designated as Buffer A and eluted (2 ml fractions) with the same buffer. The fractions, exhibiting  $\alpha$ -amylase activity were pooled; the enzyme was reprecipitated at 45%  $(\text{NH}_4)_2\text{SO}_4$  saturation and centrifuged (12,000 *g*). The pellet was dissolved and dialysed in the 0.05 M Tris buffer (pH 8.2). The dialysate was applied on Bio-gel P-100 column (2.5 cm

55 cm) and eluted (14 ml/h) with the same buffer. Fractions (3 ml each) showing zyme activity were separated into two distinct protein peaks. These were pooled separately and the enzyme protein in each peak was precipitated and dialysed as described above. The molecular weights of the individual enzymes designated as amylases I and II, were determined by calibrating Bio-gel P-100 column with the following standard proteins: albumin (68,000), ovalbumin (45,000), pepsin (35,000) and ymotrypsin (22,500). The purified enzyme samples could be stored at 5°C up to three months, without any appreciable loss of activity.

#### *Electric focusing*

The enzyme was applied on to an isoelectrofocusing column (LKB 8101, 110 ml) containing 5 to 50% sucrose gradient and 1% ampholite (pH 4 to 7) as described by Chahar and Wakil (1981). The run was continued for 66 h at 4°C with a constant stage of 500 volts. The enzyme activity and pH were measured in each fraction (2 ml). pH was determined with a EMCO digital pH meter type EE 330 A, equipped with a reference glass electrode.

#### *Starch assay*

Enzyme preparation (1 ml) was incubated with 0.15% starch in 0.01 M Na acetate buffer (pH 5.6) containing 0.003 M  $\text{Ca}^{2+}$  for 10 min at 37°C (Jones and Varner, 1967). The reaction was stopped by addition of 1 ml of  $\text{I}_2/\text{KI}$  reagent. The absorbance was measured at 620 nm in Bosch and Lomb Spectronic 20. The activity of the enzyme required for reduction of 10% in absorbance at 620 nm, was expressed as one unit.

#### *Polyacrylamide gel electrophoresis (PAGE)*

$\alpha$ -amylase isoenzymes were separated by electrophoresis on 7% polyacrylamide gels in 0.01 M Tris-glycine (pH 8.2) buffer (Davis, 1964). The protein bands were stained with Coomassie brilliant blue. The enzyme activity in different bands was detected by staining the gel for 1 h at 37°C in 1% starch solution in acetate buffer (pH 5.6) and stained with 0.003 M  $\text{I}_2$  solution.

In another experiment, the enzyme sample (Ca. 200  $\mu\text{g}$  protein) was mixed thoroughly with 1% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol and 0.1% bromophenol blue (Laemmli, 1970). A drop of glycerol was added and the sample loaded on 10% polyacrylamide gel and electrophoresed at 25°C for 2.5 h. After removal of SDS (Leach *et al.*, 1980), the gels were stained separately for proteins and carbohydrates with 0.1% Coomassie brilliant blue and fuschine sulphite blue, respectively. The bands were scanned in Shimadzu spectrophotometer at 660 nm. Molecular weights were determined by comparing their mobilities with those of marker proteins. Total proteins were estimated using crystalline BSA as standard (Biochem. J., 1959).

*Carbohydrate analysis*

Total sugars were estimated colourimetrically (Dubois *et al.*, 1956). For individual sugar analysis, the enzyme sample was hydrolysed in 3 N HCl for 4 h at 100°C and treated with Dowex-1-X8 ( $\text{HCO}_3^-$ ) to raise the pH to 7.0. The filtrate was then passed through a Dowex-50  $\text{H}^+$  column (9 cm  $\times$  1 cm). The neutral sugars were eluted with distilled water, concentrated and separated by descending paper chromatography technique with *n*-butanol:pyridine:water (6:4:3) as the solvent system. Sugars were detected by a dipping method with silver nitrate-sodium hydroxide reagents (Trevelyan *et al.*, 1950). Acidic sugars were eluted with 2 N HCl; acid was evaporated and the residue was taken in citrate buffer (pH 2.2). Glucosamine and galactosamine were analysed by a Beckman automatic amino acid analyser using reference standards.

**Results***Purification of  $\alpha$ -amylase*

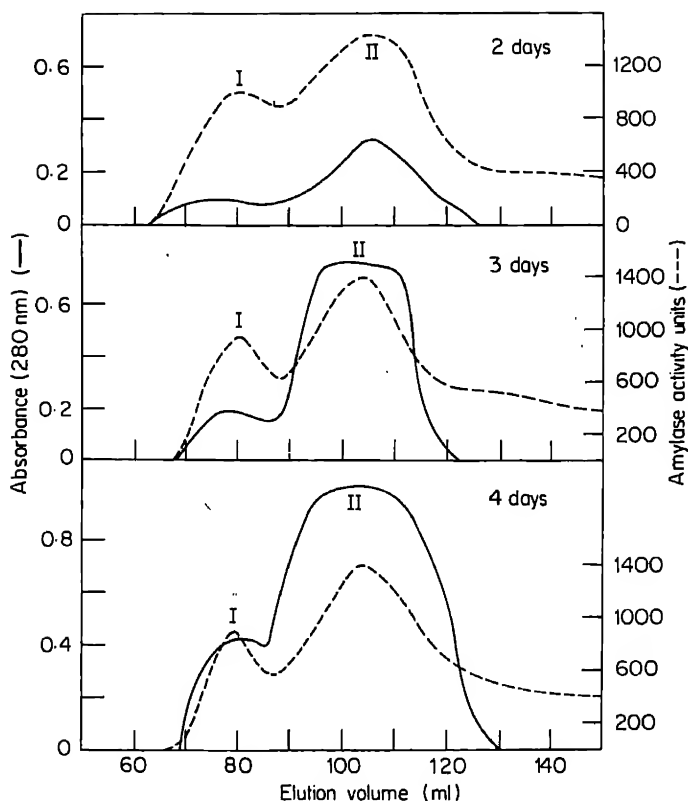
Though glycogen precipitation method (Loyter and Schramm, 1962) is widely used for the isolation of  $\alpha$ -amylase, complete removal of glycogen after the dissociation of the enzyme from the complex could not be achieved. Hence, the modified purification procedure (table 1) was followed. On addition of  $(\text{NH}_4)_2\text{SO}_4$  some interfering phenolic compounds were coprecipitated along with the enzyme. Incorporation of 1 % Polyvinyl pyrrolidone in the extraction buffer and two subsequent  $(\text{NH}_4)_2\text{SO}_4$  precipitations removed these compounds. Further, by employing Sepharose 4B chromatography,

**Table 1.** Purification of  $\alpha$ -amylases from germinating wheat.

Purification step	$\alpha$ -Amylase total units/g wheat	Specific activity units/mg protein	Purification fold
Crude extract	2,833	54	1.00
Heat treated supernatant	2,763	112	2.05
1st $(\text{NH}_4)_2\text{SO}_4$ precipitation	2,520	580	10.62
2nd $(\text{NH}_4)_2\text{SO}_4$ precipitation	2,402	770	14.10
Sepharose-4B column	2,351	1007	18.65
Bio-gel P-100	114	745	54.70*
Peak I			
Peak II	1,398	2244	

Endosperms from 4 days' old wheat seedlings were used for the isolation of the enzyme.

\* Calculated by combining the values of total protein and  $\alpha$ -amylase activities in peaks I and II after Bio-gel P-100 chromatography.



**Figure 1.** Separation of  $\alpha$ -amylases I and II by Bio-gel P-100 column chromatography.  $\alpha$ -Amylase was isolated from 2 to 4 days old wheat seedlings and subjected to gel filtration on Bio-gel P-100 column. In each fraction the protein content (—) and  $\alpha$ -amylase activity (---) were determined.

repeated dialysis and by gel filtration, the enzyme could be purified about 55-fold. This was resolved by Bio-gel P-100 column chromatography into two major distinct enzyme protein peaks referred to as  $\alpha$ -amylase I and  $\alpha$ -amylase II (figure 1). It was observed (table 2) that the enzyme activity was mainly confined to  $\alpha$ -amylase II.  $\alpha$ -Amylase I activity was reduced from about 22 to 15% during 4 days of germination with concomitant increase in  $\alpha$ -amylase II activity from 77 to 85%. Molecular weights, calibrated from Bio-gel P-100 column revealed that  $\alpha$ -amylase I had higher molecular weight (43,000) compared to that of  $\alpha$ -amylase II (22,500).

#### *Isoelectric point*

Isoelectric focusing patterns of  $\alpha$ -amylases I and II, are shown in figure 2. The single peak obtained for both the isoenzymes indicated that each component was purified to near homogeneity. These results further support the validity of comparing the physicochemical properties of the enzymes.  $\alpha$ -Amylase I had initially higher isoelectric

**Table 2.** Distribution of  $\alpha$ -amylases I and II activity in germinating wheat seedlings.

Germination time (days)	Distribution of the enzyme activity (%)	
	$\alpha$ -Amylase I	$\alpha$ -Amylase II
2	22.4 (308)	77.6 (690)
3	18.5 (555)	81.5 (1257)
4	14.9 (745)	85.1 (2244)

Distribution of the total activity in  $\alpha$ -amylases I and II was calculated (from figure 1), by measuring the peak area with planimeter. Specific activities (units/mg protein) are given in parenthesis.

point and more acidic component (pH 5.8) appeared only on the fourth day germination. Whereas, in the electrophoretic mobility of  $\alpha$ -amylase II did not vary significantly during 4 days of germination.

#### *Separation of $\alpha$ -amylase by SDS-PAGE*

Separation pattern of  $\alpha$ -amylases I and II (from 2 to 4 days old seedlings) by SDS-PAGE and the densitometric scannings of the bands are shown in figure 3. Both were resolved into 3 subunits (stained for proteins) of varying intensities with molecular weights of 22,500, 44,000 and 55,000 (peaks 1, 2 and 3, respectively). Whereas  $\alpha$ -amylase II from 4th day sample had an additional band (76,000, peak 4). However, in all the samples, maximum amount of protein as well as the enzyme activity were confined in the fast moving low molecular weight (22,500) band; though other bands were almost inactive. However, high molecular weight bands (55,000 and 76,000) of  $\alpha$ -amylase II from 3 and 4 days old seedlings, exhibited some enzyme activity (data not shown).

#### *Separation of $\alpha$ -amylase isoenzymes*

$\alpha$ -Amylases were separated into isoenzymes by simple PAGE and stained for proteins (figure 4A). Densitometric scanning of the gels are shown in figure 4B. Both  $\alpha$ -amylases I and II showed nearly the same zymogram patterns. However, significant differences in their appearance during germination were observed.  $\alpha$ -Amylase I isoenzymes (A, 1,2) were not readily distinguishable upto 3 days. However, on the 4th day, it was separated into 3 isoenzymes, (A,3; B,4I). On the other hand,  $\alpha$ -amylase II could be separated into four isoenzymes but with very low intensity on the 2nd day (A,4; B,2II). It showed a progressive increase during germination (A,5,6; B,3II, 4II). The typical distribution of total proteins and the enzyme activities in different isoenzymes on the fourth day of germination are shown in figure 5. Quantitative analysis revealed that maximum amount of proteins and enzyme activities could be recovered from the most electropositive and fastest moving 4th band of  $\alpha$ -amylase II (2) sample. Other isoenzyme bands had low activity.



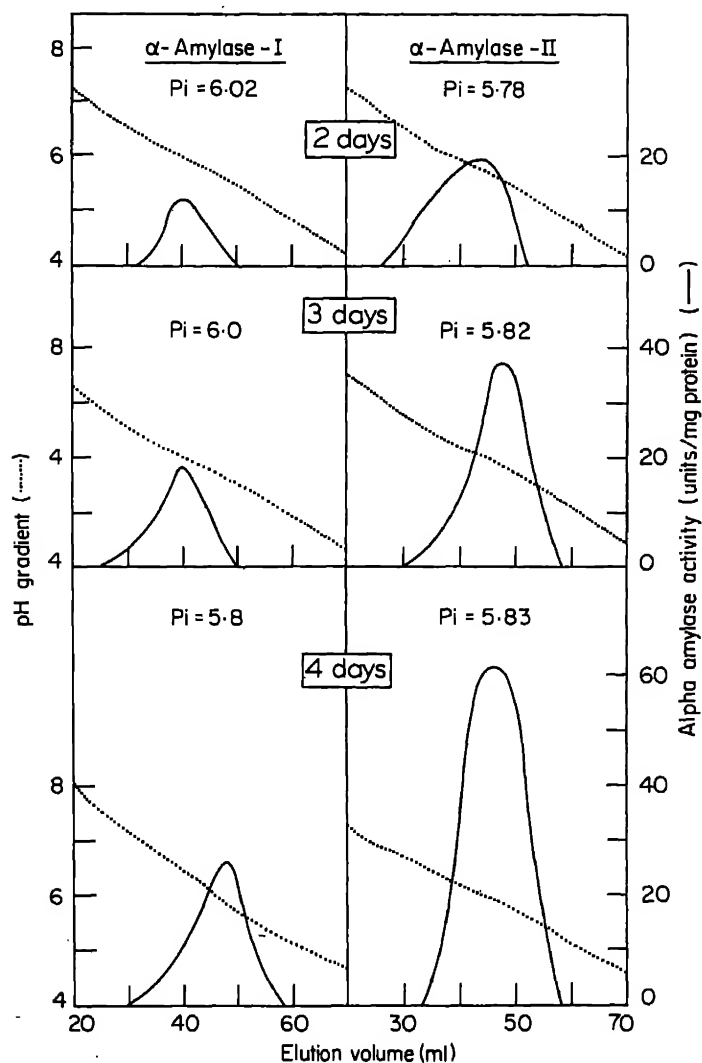


Figure 2. Isoelectric focusing of  $\alpha$ -amylases I and II purified from germinating wheat.  $\alpha$ -Amylase (200  $\mu$ g) was electrofocused as described in the text. After 66 h run, 1 ml fractions were collected and the enzyme activity (—) and pH (.....) determined in each fraction.

#### Carbohydrate content of $\alpha$ -amylases

It was observed that  $\alpha$ -amylase bands, separated by SDS-PAGE, were positively stained for carbohydrates (figure 3, bottom gel). In both,  $\alpha$ -amylases I and II, the maximum staining was in 22,500 band (number 1), whereas other bands of higher molecular weights were faintly stained. Total sugar content of 22,500 dalton bands was always higher in  $\alpha$ -amylase I than in II, but decreased as the germination proceeded

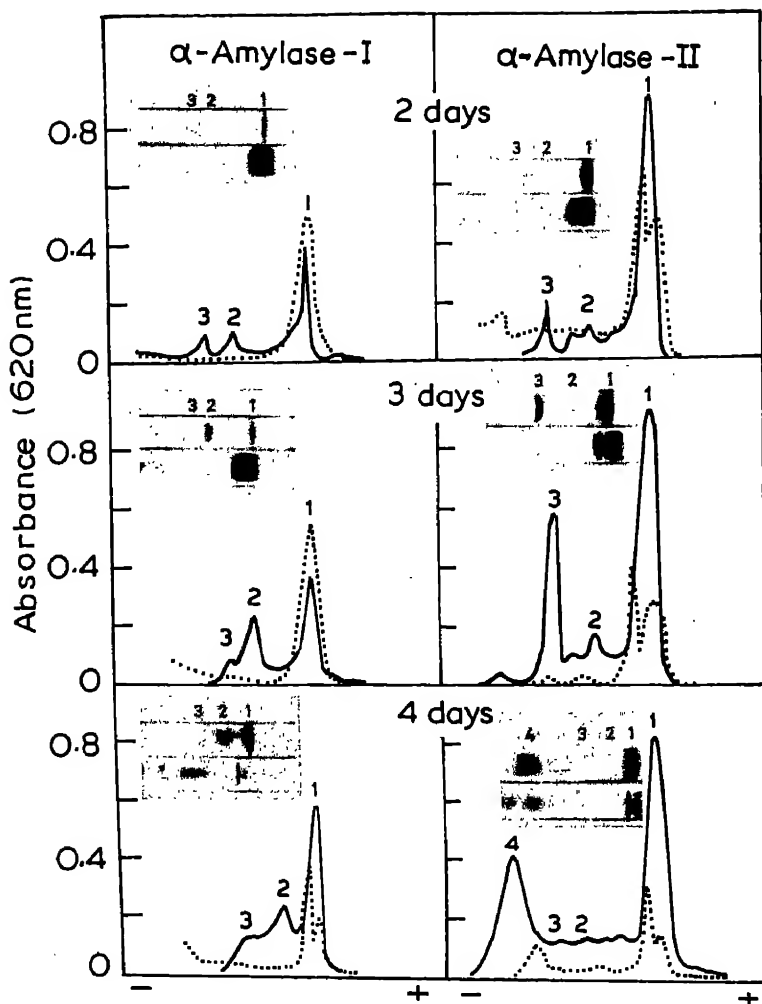


Figure 3. SDS-PAGE of  $\alpha$ -amylases I and II from germinating wheat. Electrophoresis was carried out and the gels were stained for proteins (top) and for carbohydrates (bottom) as described in the text. These were then scanned for proteins (—) and carbohydrates (....) and absorbancy at 620 nm was measured.

(table 3). In all  $\alpha$ -amylase I samples, carbohydrates, were confined, almost 100% to 22,000 protein. However, in  $\alpha$ -amylase II, 22,000 protein contained 90.5, 93.8 and 81.3% of total sugars from 2, 3 and 4 days old wheat seedlings, respectively. This gradual decrease was reflected in concomitant increase in carbohydrate contents in 44,000 (2.5 to 5.2%) and 55,000 (3.7 to 4.3%) proteins from 2nd and 3rd days.  $\alpha$ -Amylase II samples similarly, 76,000 polypeptide from the 4th day sample contained about 18.5% carbohydrate. Further, carbohydrates:protein ratio was calculated. On the fourth day, when the enzyme activity was maximum (table 2), the ratio was 1:31 in  $\alpha$ -amylase I and

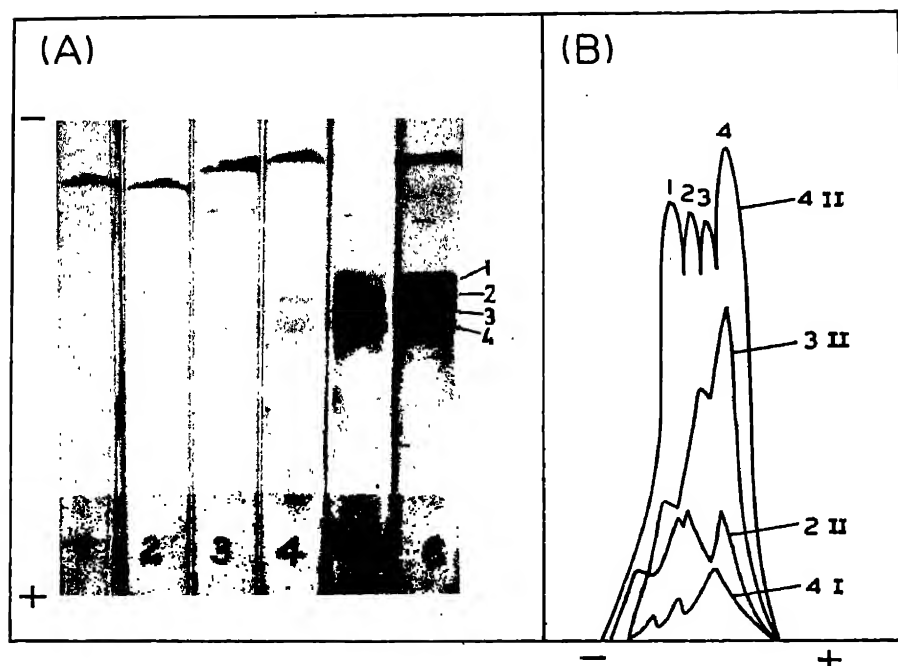


Figure 4. Separation of  $\alpha$ -amylase isoenzymes by PAGE.  $\alpha$ -Amylase isoenzyme bands (1 to 4 on gels) were stained for proteins as described in the text. 1,2,3:  $\alpha$ -amylase I zymograms and 4,5,6:  $\alpha$ -amylase II zymograms at 2, 3 and 4 days germination, respectively. Densitometric scanning (B) of  $\alpha$ -amylase I gel at 4 days' (4 I),  $\alpha$ -Amylase II gels (2 II; 3 II and 4 II) at 2, 3 and 4 days' germination, respectively.

only 0.14 in  $\alpha$ -amylase II, the most active enzyme form. This indicates an inverse relationship between this ratio and the enzyme activity.

Further, the enzyme samples from wheat seedlings were hydrolysed and separated into individual sugars by paper chromatography (figure 6). Since xylose and fucose were cochromatographed by the method employed, the corresponding spot was tested chemically for fucose using cysteine-sulphuric acid reaction (Dische and Shettles, 1948). Fucose was not detected in any of the samples analysed. The quantitative analysis of neutral sugars (table 4) revealed that xylose and mannose were the main sugars. They contributed about 25 to 30% and 45 to 50%, of the respective content of total sugars in  $\alpha$ -amylases I and II. It was interesting to note that on the fourth day in  $\alpha$ -amylase II, galactose was not detected but glucose content (25%) was higher than that in  $\alpha$ -amylase I (13%). Galactosamine was not present in any of the samples. Glucosamine was present only in the 4th day samples;  $\alpha$ -amylase II contained higher amount (10  $\mu\text{g}/\text{mg}$  protein) compared to that in  $\alpha$ -amylase I (4.5  $\mu\text{g}/\text{mg}$  protein).

## Discussion

Total  $\alpha$ -amylase activity as well as protein content in wheat seedlings were maximum on the fourth day. However, this was reflected mainly by a marked increase in  $\alpha$ -amylase II

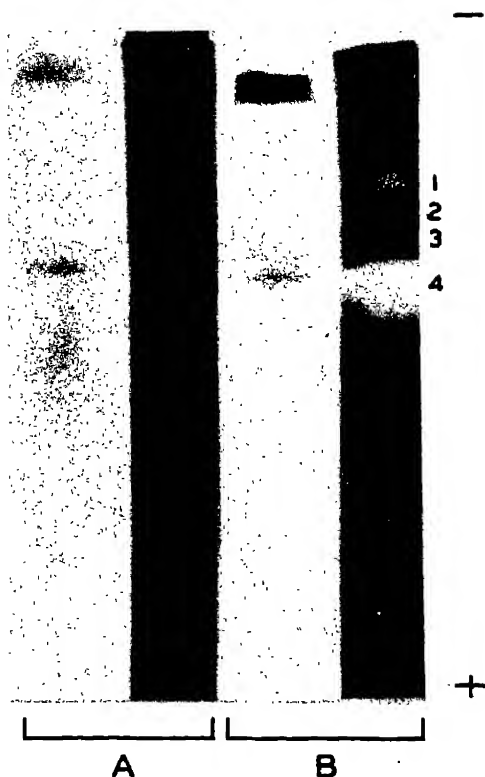


Figure 5. Electrophoretic patterns of  $\alpha$ -amylases at 4 days of germination.  $\alpha$ -Amylases I (A) and II (B) were separated by gel-electrophoresis. Gels were incubated with starch solution as described in the text. Four bands with  $\alpha$ -amylase activity were obtained.

Table 3. Determination of carbohydrate: protein ratio of  $\alpha$ -amylases (22,000 band).

Germination (days)	Carbohydrate:protein ratio	
	$\alpha$ -Amylase I	$\alpha$ -Amylase II
2	2.42 (15)	1.00 (1.2)
3	2.22 (11.1)	0.68 (1.5)
4	1.31 (9.2)	0.14 (2.6)

The areas of peaks related to carbohydrate and proteins of 22,500 bands (figure 3) were measured and their ratio calculated. The values were averages of two independent experiments in duplicates. The figures in the parenthesis total carbohydrates (mg/100 mg of protein).

activity compared to  $\alpha$ -amylase I (figure 1). Similar differences were also observed when sequential occurrence of their isoenzyme patterns were examined by PAGE (figure 5). The low enzyme activity in  $\alpha$ -amylase I could be attributed to a delayed appearance of the most electropositive fast-moving band; which appeared earlier in  $\alpha$ -amylase II. Molecular weights were estimated as 43,000 and 22,500, respectively for  $\alpha$ -amylase I

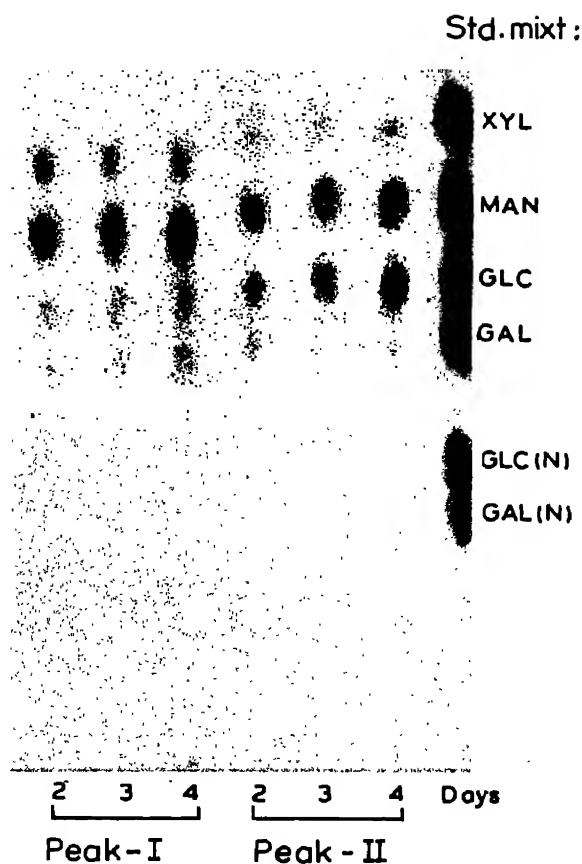


Figure 6. Separation of neutral sugars of  $\alpha$ -amylases. Purified enzyme preparations from 2, 3 and 4 days old wheat seedlings were subjected to paper chromatography. Neutral sugars were detected as described in the text.

Table 4. Carbohydrate composition of wheat  $\alpha$ -amylases I and II.

Germination (days)	$\alpha$ -Amylase	Neutral sugars ( $\mu$ g/mg protein)			
		Xylose	Mannose	Glucose	Galactose
2	I	35.3 (28.6)	54.4 (44.0)	22.0 (17.8)	11.8 (9.6)
	II	2.9 (24.7)	3.7 (48.0)	1.4 (18.2)	0.7 (9.1)
3	I	25.7 (28.3)	42.4 (46.7)	16.6 (18.3)	6.1 (6.7)
	II	3.5 (28.0)	6.6 (50.4)	3.0 (21.6)	—
4	I	24.6 (31.6)	36.9 (47.4)	10.2 (13.2)	6.1 (7.9)
	II	6.3 (29.2)	9.9 (45.8)	5.4 (25.0)	—

Neutral sugars were separated from acid hydrolysate of the enzyme samples by paper chromatography and quantified colorimetrically, using glucose as standard. The values are mean of three experiments in duplicates. Figures in the parenthesis represent % of the total sugars present.

and II. They showed a tendency to aggregate in the presence of SDS. However, these high molecular weight bands may not be artifacts, since high molecular weight bands of  $\alpha$ -amylase II (figure 3, bands 3 and 4) were enzymically active. The anomalous behaviour of glycoproteins in the presence of SDS is known, and the glycopeptide-SDS complexes yield abnormally high molecular weight estimates (Leach *et al.*, 1980). Similarly, isoelectric points of  $\alpha$ -amylases I and II, separated from 2 and 3 days old seedlings were different. Such differences in isoelectric pH of  $\alpha$ -amylase components at different stages of barley germination had been reported (MacGregor, 1978).

Further, a correlation between the enzyme activity and the sugar content was observed. Further,  $\alpha$ -amylases I and II were heterogenous in their sugar content (table 4). Such heterogeneity with respect to sugar content in barley  $\alpha$ -amylases had also been shown (Rodaway, 1978). Noticeably,  $\alpha$ -amylase I contained very high proportion of mannose units. Several secretory glycoproteins from animal and plant cells contain high mannose type oligosaccharides (Bailey *et al.*, 1980). The rapid disappearance of carbohydrate moieties of  $\alpha$ -amylase II as the germination proceeds, also suggests the rapid turnover of sugar residues than that of the polypeptides of the enzyme.

It has been shown that  $\alpha$ -amylase is synthesised on rough endoplasmic reticulum (Jones and Chen, 1976), transported to the lumen of endoplasmic reticulum and glycosylated (Czichi and Lennars, 1977). This is followed by their accumulation in the secretory vesicles (Gibson and Paleg, 1975). Miyata *et al.* (1981) demonstrated that an oligosaccharide chain is added to the cleaved polypeptide precursor of the enzyme in germinating rice to produce the mature and final secretory form of the enzyme. Since only deglycosylated protein exhibits maximum activity, we are tempted to suggest that  $\alpha$ -amylase I represents the secretory protein synthesized and fully glycosylated; and  $\alpha$ -amylase II is the secreted active form of the enzyme, variously deglycosylated on different days of germination.

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## Water stress induced alterations in ornithine aminotransferase of ragi (*Eleusine coracana*): Protection by proline against heat inactivation and denaturation by urea and guanidinium chloride

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**Abstract.** Water stress resulted in a specific response leading to a large and significant increase (80-fold) in free proline content of ragi (*Eleusine coracana*) leaves and seedlings. L-Proline protected ornithine aminotransferase, an enzyme in the pathway for proline biosynthesis, isolated from normal and stressed ragi leaves against heat inactivation and denaturation by urea and guanidinium chloride. The protection of the stressed enzyme by L-proline was much more complete than that of the enzyme isolated from normal leaves. While L-ornithine, one of the substrates, protected the stressed enzyme against inactivation, it enhanced the rate of inactivation of the normal enzyme.  $\alpha$ -Ketoglutarate protected both the normal and stressed enzyme against inactivation and denaturation. These results support the suggestion that ornithine aminotransferase has undergone a structural alteration during water stress. In view of the causal relationship between elevated temperature and water stress of plants under natural conditions, the protection afforded by proline against inactivation and denaturation of the enzyme from stressed leaves assumes significance. These results provide an explanation for a possible functional importance of proline accumulation during water stress.

**Keywords.** Ornithine aminotransferase; proline; water stress; *Eleusine coracana*.

### Introduction

The most significant and easily detectable biochemical change when plants are deprived of water is the accumulation of free proline (Singh *et al.*, 1972). In an earlier study, we showed that in polyethyleneglycol (PEG)-treated ragi (*Eleusine coracana*) leaves, the level of free proline increased by 85-fold and this increase was probably due to an enhanced activity of the enzymes involved in the synthesis of proline and marked inhibition of the activity of enzymes degrading proline (Kandpal *et al.*, 1981). The activity of ornithine aminotransferase (EC 2.6.1.13), an enzyme of proline biosynthesis, increased by about 3 fold in water-stressed ragi leaves. In a subsequent study, we showed that the increase in the activity of ornithine aminotransferase was not due to the presence of an activator but due to alteration in the properties of the enzyme isolated from water stressed leaves (Kandpal and Appaji Rao, 1982). The present study was undertaken to determine the differences between the enzyme isolated from normal and stressed leaves by using heat inactivation and denaturation by urea and guanidinium

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Abbreviations used: PEG, Polyethyleneglycol (molecular weight 6000); GdmCl, guanidinium chloride.

chloride (GdmCl), as well as the effects of substrates and proline on inactivation patterns as probes for alterations in the structure of ornithine aminotransferase consequent to water stress.

## Materials and methods

### Materials

All biochemicals used in this study were obtained from the Sigma Chemicals Co., St. Louis, Missouri, USA. Polyethyleneglycol (6,000 molecular weight) was obtained from the SD's Lab Chem Industry, Bombay. All other chemicals and buffer components were of the reagent grade. Ragi (*Eleusine coracana*) seeds variety Purna were obtained from Dr. K. S. Krishnasastry, University of Agricultural Sciences, Bangalore.

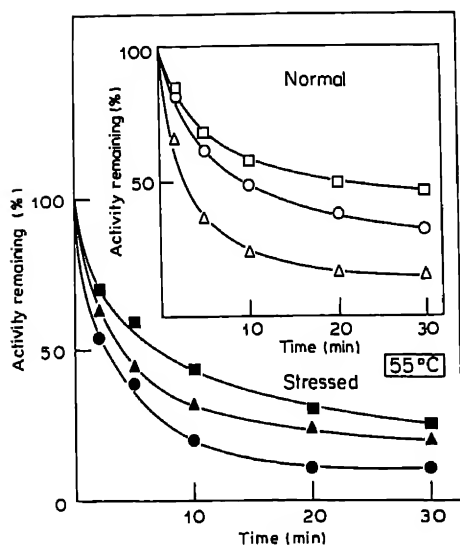
### Methods

*Induction of water stress and purification of ornithine aminotransferase:* Ragi seeds were grown in the Institute Nursery for a period of 30–45 days and young and fully expanded leaves were collected randomly and pooled for the isolation of the enzyme. In one set, the cut ends of the leaves were partly dipped in 40% PEG and illuminated for 6–8 h. The biochemical and physiological changes caused by PEG treatment were similar to natural water deprivation (Todd, 1972). It was also ascertained that in the ragi, proline accumulation, changes in enzyme activities RNA and DNA metabolism were similar in both PEG-treated and naturally water-stressed ragi leaves and seedlings (Kandpal, 1983). At the end of 8 h, the leaves were washed free of PEG, blot-dried, cut into small pieces and stored frozen at  $-20^{\circ}\text{C}$ . The normal leaves were similarly treated with water and processed. Acetone powder of the normal and PEG-treated leaves was prepared separately. The enzyme was partially purified from both the normal and stressed leaves and activity assayed as described earlier (Kandpal and Appaji Rao, 1982).

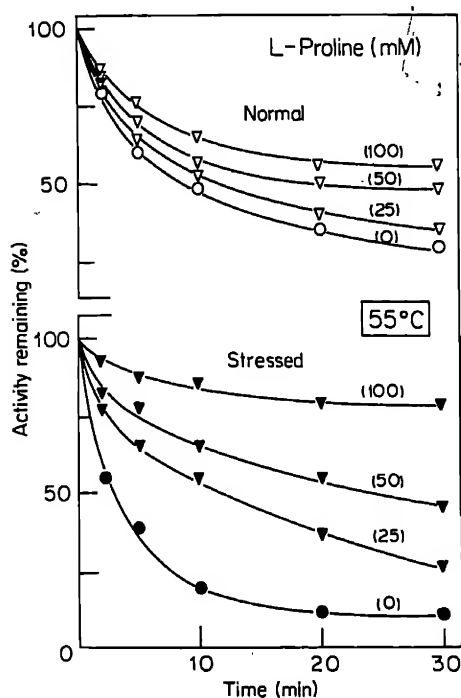
## Results

### *Effect of substrates and proline on thermal stability of ornithine aminotransferase isolated from normal and stressed ragi (Eleusine coracana) leaves*

It is evident from figure 1 that the normal enzyme retained about 40% of the original activity at the end of 30 min heating at  $55^{\circ}\text{C}$ . The stressed enzyme, on the other hand, lost about 90% of its activity in this time interval. The stressed enzyme was protected to the extent of 20 and 30% respectively by L-ornithine (12.0 mM) and  $\alpha$ -ketoglutarate (15.0 mM). In the case of the normal enzyme, while  $\alpha$ -ketoglutarate offered significant protection ( $t_{1/2} = 20$  min), L-ornithine enhanced ( $t_{1/2} = 4$  min) its rate of inactivation



**Figure 1.** Heat inactivation patterns of the normal and stressed ornithine aminotransferase. The normal (O) or stressed (●) enzymes (5 mg/ml) in 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) were heated at 55°C in the absence of any of the substrates (O, ●) and in the presence of 12.0 mM L-ornithine (Δ, ▲) and 15.0 mM α-ketoglutarate (□, ■). Aliquots of 0.1 ml of the enzyme were withdrawn at time points indicated in the figure and rapidly cooled. The enzyme activity was assayed at pH 8.0 and 37°C (Kandpal and Appaji Rao, 1982). The enzyme activity at zero time was normalized to 100 and the residual activity expressed as percent of this control.



**Figure 2.** Protection by L-proline against heat inactivation of the normal and stressed ornithine aminotransferase. The normal (O) or stressed (●) enzymes (5 mg/ml) in 0.1 M potassium phosphate buffer (pH 8.0) were heated at 55°C in the absence (O, ●) and in the presence of 25 mM, 50 mM and 100 mM L-proline (V, ▼). Aliquots of 0.1 ml were withdrawn at time points indicated in the figure and rapidly cooled. L-Proline (100 mM) had no effect on the catalytic activity of either normal or stressed enzyme.

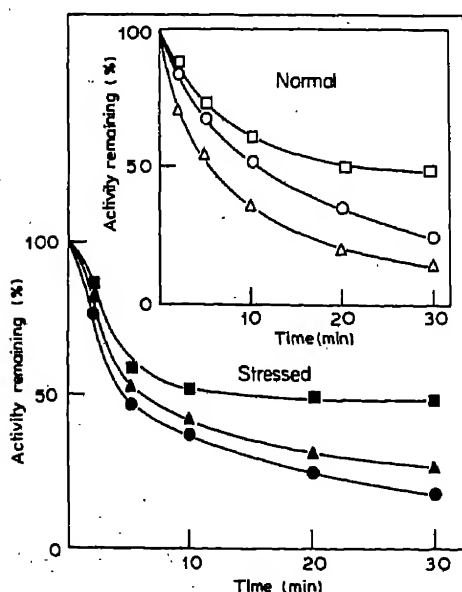
(figure 1). Proline protected the normal and stressed enzyme in a concentration-dependent manner, although the extent of protection was different (figure 2). At a maximal concentration of 100 mM, proline protected the normal enzyme only to 55% whereas the stressed enzyme was almost completely (80%) protected against heat inactivation at 55°C for 30 min (figure 2).

*Effect of substrates and proline on urea and GdmCl denaturation of the enzyme isolated from normal and stressed ragi leaves*

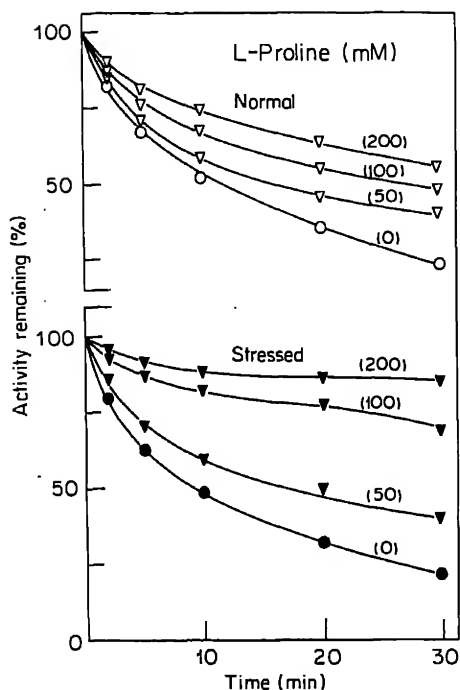
Although the denaturation patterns of the normal and stressed enzyme did not differ markedly in the absence of substrates, the differences were discernible when denaturation was carried out in the presence of substrates. While  $\alpha$ -ketoglutarate had comparable protective effect on the normal and stressed enzyme, L-ornithine enhanced the denaturation of the normal enzyme and protected the stressed enzyme by about 40% (figure 3).

The protection by proline against urea denaturation is shown in figure 4. The effect of proline on urea denaturation patterns of the enzyme is similar to that against heat inactivation, although a higher concentration of proline was required for appreciable protection. At maximal concentration of proline (200 mM), the normal and stressed enzyme retained 70 and 90% activity respectively (figure 4).

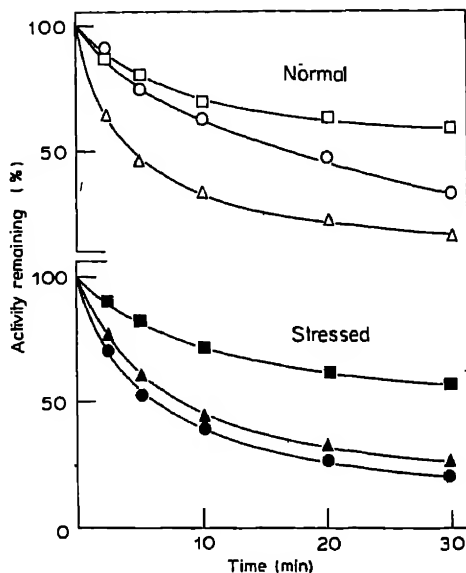
The enzymes from normal and stressed leaves were not inactivated at similar rates by GdmCl (0.5 M), the stressed enzyme being inactivated at a much faster rate. While L-ornithine enhanced the rate of inactivation of the normal enzyme, it was without effect or provided marginal protection against inactivation of the stressed enzyme. The rates of denaturation of the normal and stressed enzyme in the presence of  $\alpha$ -ketoglutarate were similar (figure 5). The stressed enzyme which was being rapidly inactivated by GdmCl, was very significantly protected by L-proline in a concentration-dependent manner. The effect of proline on the normal enzyme was similar but the extent of protection was not as marked (at 10 min, the stressed enzyme in the presence of 100 mM proline had 85% activity compared to 45% in the absence of proline, whereas the values for the normal enzyme were 80 and 65% respectively, figure 6).



**Figure 3.** Urea denaturation patterns of normal and stressed ornithine aminotransferase. The normal (O) or stressed (●) enzymes (5 mg/0.5 ml of buffer pH 8.0) were incubated separately with 0.5 ml of 2 M urea solution (pH 8.0) at 37°C in the absence of any of the substrates (O, ●) and in the presence of 12.0 mM L-ornithine (Δ, ▲) and 15.0 mM  $\alpha$ -ketoglutarate (□, ■). Aliquots of 0.1 ml were withdrawn at time points indicated in the figure.



**Figure 4.** Protection by L-proline against urea denaturation of normal and stressed ornithine aminotransferase. The normal (O) or stressed (●) enzymes (5 mg/0.5 ml of buffer pH 8.0) were incubated separately with 0.5 ml of 2 M urea solution (pH 8.0) at 37°C in the absence (O, ●) and in the presence of 50 mM, 100 mM and 200 mM proline (V, ▼). Aliquots of 0.1 ml were withdrawn at time points indicated in the figure.



**Figure 5.** GdmCl denaturation patterns of normal and stressed ornithine aminotransferase. The normal (O) or stressed (●) enzymes (5 mg/0.5 ml of buffer) were incubated separately with 0.5 ml of 1 M GdmCl at 37°C in the absence (O, ●) of any of the substrates and in the presence of 12.0 mM L-ornithine (Δ, ▲) and 15 mM α-ketoglutarate (□, ■). Aliquots (0.1 ml) were withdrawn at time points indicated in the figure.

## Discussion

The dramatic accumulation of proline in water-stressed plants has been ascribed to enhanced rate of synthesis due to increased activity of the enzymes involved in the biosynthesis and marked inhibition of the enzymes responsible for its degradation (Kandpal *et al.*, 1981). Our earlier results showed that changes in the activity of ornithine aminotransferase were not due to the presence of a 'factor' which regulated its properties (Kandpal and Appaji Rao, 1982). Differential rates of inactivation by denaturing agents on interaction with small molecular weight ligands have been extensively used to indicate altered structures in protein molecules (Kaufman, 1968; Citri, 1973). Our results (figure 1) show that the stressed enzyme is more rapidly inactivated by heat compared to the enzyme from normal leaves. L-Ornithine, one of the substrates, protected the stressed enzyme while it enhanced the inactivation of the normal enzyme suggesting that water stress might have altered the structure of the

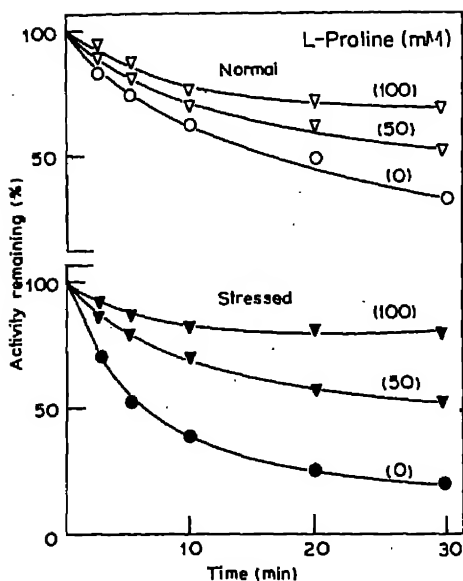


Figure 6. Protection by L-proline against GdmCl denaturation of normal and stressed ornithine aminotransferase. The normal (O) or stressed (●) enzymes were incubated separately with 0.5 ml of GdmCl at 37°C in the absence (O, ●) and presence of 50 mM and 100 mM proline (□, ■). Aliquots of 0.1 ml were withdrawn at time points indicated in the figure.

enzyme. Similar denaturation profiles in the presence and absence of ligands were obtained when denaturation was carried out at 50° and 60°C (data not given). Further support to this hypothesis is lent by urea and GdmCl denaturation patterns of the normal and stressed enzyme in presence of L-ornithine (figures 3 and 5).

In view of the large accumulation of proline during stress (Chu *et al.*, 1978; Hellebust, 1976), and the effects of proline on protein structure due to its hydrogen bonding capacity and interactions with hydrophobic regions in the protein (Schobert, 1977; Schobert and Tschesche, 1978) it was of interest to examine the effect of proline on an enzyme involved in its biosynthesis. As high as 200 mM concentration of L-proline had no stimulatory or inhibitory effect on the enzyme activity. It is significant that although proline afforded some protection to the normal enzyme, it almost completely protected the stressed enzyme against heat inactivation and urea and GdmCl denaturation. This is especially pertinent as elevation in temperature occurs during water stress which may result in inactivation of enzymes (Smith, 1978). The stressed enzyme which encounters high temperature is also protected by proline which accumulates during water stress. It has been calculated that the concentration of proline in stressed tissue varies between 100 and 200 mM.

It could be suggested from these observations that inhibition of the degradation of proline during water stress (Kandpal *et al.*, 1981) resulted in its accumulation, which in turn by its protective effects on the enzymes involved in its biosynthesis further facilitated marked accumulation of proline. These effects of proline provide clues to the possible role it may have in the adaptation of some plants to intermittent stress to which they are subjected when grown in the tropics.

## Acknowledgements

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## Characteristics of a nuclear protein kinase from rat epididymis

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**Abstract.** Purified rat epididymal nuclei possess a cyclic AMP-independent protein kinase activity that phosphorylates casein. The enzymic activity was solubilized by treating intact nuclei with 1 M  $(\text{NH}_4)_2\text{SO}_4$ . One major peak of kinase activity was obtained when the solubilized enzyme preparation was subjected to diethylaminoethyl-Sephadex chromatography. The activity of the kinase was dependent on a bivalent metal ion such as  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ . NaCl (0.3 M) caused a further activation (approx. 200%) of the metal ( $\text{Co}^{2+}$ )-dependent enzyme. The apparent  $K_m$  values of the enzyme for casein, ATP and  $\text{Co}^{2+}$  are approx. 0.6 mg/ml, 10  $\mu\text{M}$  and 2.2 mM respectively. The enzyme was maximally active at pH 5.5. The enzyme showed high specificity for phosphorylation of the acidic protein casein but did not phosphorylate basic proteins, such as histones and protamine. The properties of the nuclear protein kinase were clearly different from those of the cytosolic enzymes previously characterized.

**Keywords.** Protein kinase; epididymis, nuclei; protein phosphorylation.

### Introduction

Several observations on mammalian tissues other than epididymis, indicated a marked alteration in the pattern of phosphorylation of nuclear proteins consequent to hormones action (Ahmed, 1971; Jungmann *et al.*, 1974; Kadohama and Turkington, 1973). It was suggested that regulation of phosphorylation of nuclear proteins could be an important regulatory mechanism for the hormone-dependent selective expression of genes (Ahmed and Ishida, 1971; Jungmann and Schweppe, 1972; Kliensmith, 1975; Jungmann and Russell, 1977). A large number of acidic chromatin proteins were found to be phosphorylated in rat epididymis, and the pattern of their phosphorylation was altered during epididymal development *in vivo*. These epididymal developmental changes were dependent on the action of testosterone (Kadohama and Turkington, 1974). However, very little is known about the mechanism of the testosterone-dependent alteration of the phosphorylation of the epididymal acidic chromatin proteins. Previous studies from this laboratory characterized two isoenzymes of cyclic AMP-dependent protein kinase in rat epididymal cytosol. Both the enzymes showed high specificity for phosphorylating basic proteins, histones and protamine rather than the acidic proteins, casein and phosvitin (Biswas and Majumder, 1982). The present study was undertaken to elucidate the enzymic characteristics of the rat epididymal

Abbreviation used: DEAE, Diethylaminoethyl.

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nuclear protein kinase that might play a pivotal role in the phosphorylation of nuclear proteins.

## Materials and methods

### Chemicals

[ $^{32}\text{P}$ ]-Orthophosphoric acid was purchased from Bhaba Atomic Research Centre, Bombay. Diethylaminoethyl-(DEAE)-Sephadex was a product of Pharmacia, Uppsala, Sweden. Adenosine 3'5'-monophosphate (cyclic AMP), calf thymus whole histones and phosphovitin were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Casein was generously supplied by Prof. N. C. Ganguli of National Dairy Research Institute, Karnal. [ $\gamma$ - $^{32}\text{P}$ ]-ATP was prepared by the method described by Majumder and Biswas (1979).

### Isolation of epididymal nuclei

Epididymides from adult rats were dissected out, minced thoroughly and dispersed in 0.25 M sucrose-0.01 M Tris-HCl buffer pH 7.5-0.1 M KCl-0.003 M  $\text{MgCl}_2$  (Majumder, 1977) with gentle stirring until most of the spermatozoa were removed. The tissue was then homogenized in the same buffer (1 g/10 ml) and the homogenate was filtered through ten layers of cheese-cloth to remove contaminating spermatozoa (Biswas and Majumder, 1982). The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant was discarded. The resulting crude nuclear pellet was washed twice with the same buffer and then dispersed in 2 M sucrose to sediment purified nuclei by centrifugation at 40,000 *g* for 60 min (Chauveau, *et al.*, 1956). The nuclear preparation showed high degree of purity as judged by phase contrast microscopy.

### Extraction and purification of nuclear protein kinase

The purified nuclear pellet was washed with 0.14 M NaCl to remove cytoplasmic contaminants. The washed nuclei were then dispersed in 50 mM Tris-HCl buffer pH 7.5 by extensive homogenization. A saturated solution of ammonium sulphate was then added to a final concentration of 1 M. The sample was then homogenized extensively, kept in ice for 30 min and centrifuged at 27,000 *g* for 30 min. The resulting supernatant was dialysed extensively against 50 mM Tris-HCl buffer pH 8.0-10 mM KCl and then loaded onto a DEAE-Sephadex column (1.2  $\times$  7.0 cm) previously equilibrated with the same buffer. The column was washed with 20 ml of the equilibration buffer prior to elution with a linear gradient of KCl (0.01-1.0 M) in a total volume of 150 ml of buffer. Three ml fractions were collected at the rate of 20 ml/h. The fractions containing the major enzymic peak were pooled, dialysed against 50 mM Tris-HCl buffer pH 8.0-10 mM KCl-30% glycerol and preserved at  $-20^\circ\text{C}$ .

The protein content of the enzyme preparations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

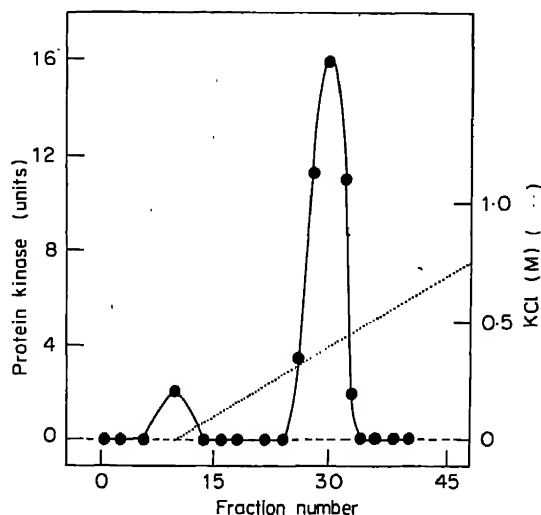
### Assay of nuclear protein kinase

The activity of nuclear protein kinase was measured by modifications of the method described earlier (Majumder, 1977). The standard reaction mixture contained 10  $\mu$ mol sodium glycerophosphate-HCl buffer pH 5.5, 2  $\mu$ mol cobalt chloride, 30 nmol EGTA, 6 nmol [ $\gamma$ - $^{32}$ P]-ATP (containing  $8 \times 10^4$ – $20 \times 10^4$  cpm), 60  $\mu$ mol sodium chloride, 2  $\mu$ mol sodium fluoride, 0.5 mg of casein and the enzyme in a total volume of 0.2 ml, and incubated out at 37°C for 20 min. The reaction was stopped by the addition of 0.2 ml of 1% casein containing 2.5 mM inorganic phosphate and 2 mM, ATP; and 4 ml 15% trichloroacetic acid. The resulting precipitate was processed for the estimation of radioactivity (Biswas and Majumder, 1982). The samples were counted in a liquid scintillation spectrometer (Packard) in a toluene–0.4% 2,5-diphenyloxazole–0.005% 1,4 bis-[2-(5-phenyloxazole)] benzene scintillation fluid. One unit of protein kinase activity was defined as the amount of enzyme which catalyses the transfer of 1 pmol [ $^{32}$ P] from [ $\gamma$ - $^{32}$ P]-ATP to the protein under the standard assay conditions. The data presented are mean values of duplicate determinations differing by less than 10%. The results are representative of at least two such experiments.

## Results

### Enzyme purification

Purified rat epididymal nuclei possess traces of protein kinase activity that phosphorylates casein. However, the enzymic activity increased markedly (approx. 4-fold)



**Figure 1.** DEAE-Sephadex chromatographic profile of rat epididymal nuclear protein kinase. Nuclear extract (0.28 mg protein) was applied to a column of DEAE-Sephadex (1.2  $\times$  7 cm). An aliquot of 50  $\mu$ l from each fraction was assayed for protein kinase activity under standard assay conditions.

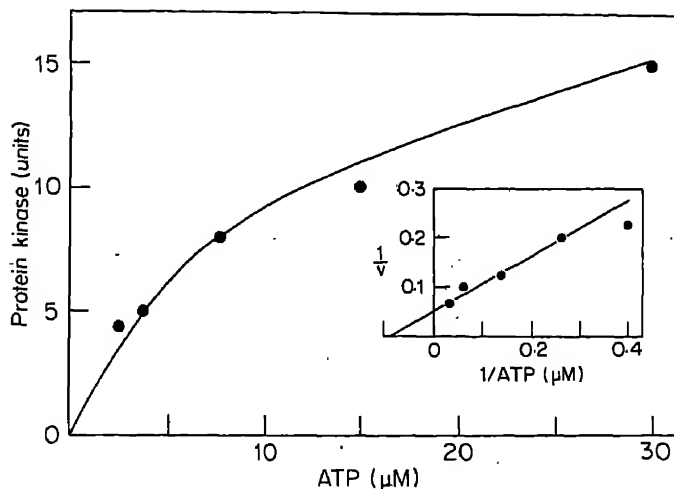
when the nuclear kinase was solubilized with 1 M  $(\text{NH}_4)_2\text{SO}_4$  (data not shown). One major peak of activity was obtained when the soluble kinase preparation was subjected to DEAE-Sephadex chromatography (figure 1). The active fractions (26–33), were pooled, dialysed against 50 mM Tris-HCl buffer pH 8.0–10 mM KCl–30% glycerol and stored at  $-10^\circ\text{C}$ . The specific activity of the major peak of activity was approx. 25,000 units/mg protein (approx. 30-fold purified) and the recovery was approx. 50%. This preparation of the enzyme was used in these studies unless otherwise stated.

### *Properties of the enzyme*

The epididymal nuclear protein kinase phosphorylating casein, was linear upto at least 25 units of the enzyme under standard assay conditions. The enzymic activity increased linearly with time for approx. 20 min. Cyclic AMP ( $1\text{ }\mu\text{M}$ ) had no effect on the activity of the enzyme indicating that the nuclear kinase was a cyclic AMP-independent enzyme (data not shown). The enzymic activity was maximal at pH 5.5 when 50 mM sodium glycerophosphate-HCl was used as the buffer. The effect of ATP concentration on the activity of the epididymal nuclear kinase is shown in figure 2. The apparent  $K_m$  value of the enzyme for ATP was found to be  $10\text{ }\mu\text{M}$ .

### *Effect of metal ions*

Nuclear protein kinase was inhibited completely when the bivalent metal ion ( $\text{Co}^{2+}$ ) was omitted from the assay (table 1), indicating that the enzymic activity was dependent on a bivalent metal ion.  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  or  $\text{Ca}^{2+}$  were found to activate the enzyme, while  $\text{Zn}^{2+}$  was without effect.



**Figure 2.** Effect of ATP concentrations on the activity of nuclear protein kinase. Insert: The Lineweaver-Burk plot of the data.

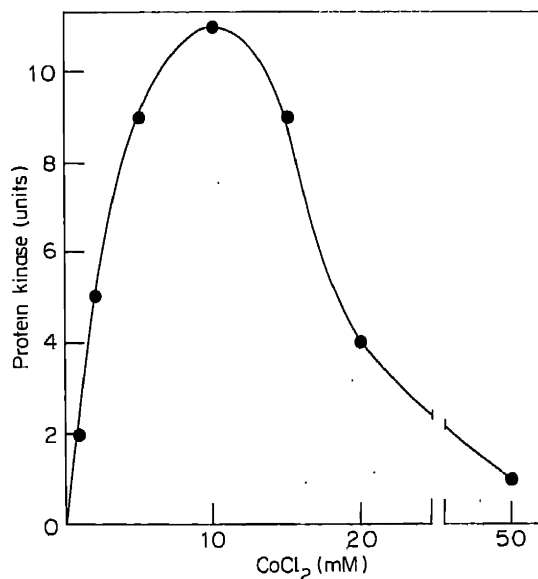
**Table 1.** Effect of metal ions on the activity of the epididymal nuclear protein kinase.

Metal ion	Protein kinase activity (units)
Null (control)	0
+ MgCl (10 mM)	38
+ CoCl (10 mM)	30
+ MnCl (10 mM)	28
+ ZnCl (2 mM)	0
+ CaCl (10 mM)	14

Standard assay system was used except for the replacement of  $\text{Co}^{2+}$  by the specified metal ions. The control contains all the reactants as defined in the standard assay except that  $\text{Co}^{2+}$  was omitted.

The effect of varying concentrations of  $\text{Co}^{2+}$  on the nuclear kinase activity is shown in figure 3. Maximal stimulation of the enzymic activity by the metal ion was observed at a concentration of 10 mM. Further increase of the metal ion concentration caused marked inhibition of the activity of the enzyme. A similar pattern was also obtained when varying concentrations of  $\text{Mg}^{2+}$  were used to activate the enzyme (data not shown). The apparent  $K_m$  values of the kinase for  $\text{Co}^{2+}$  and  $\text{Mg}^{2+}$  were 2.2 and 3.0 mM, respectively (calculated on the basis of a direct plot of the data).

NaCl (0.3 M) could not stimulate significantly the activity of protein kinase in the absence of a bivalent metal ion (table 2). However, NaCl (0.3 M) markedly stimulated

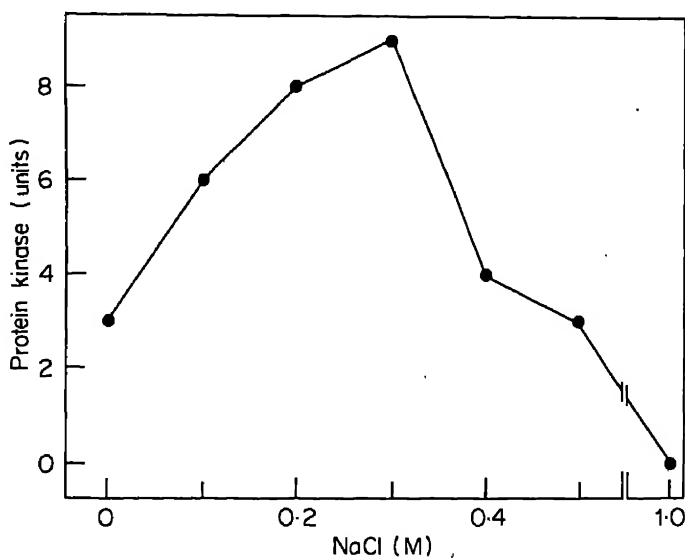
**Figure 3.** Effect of various concentrations of cobalt chloride on the activity of nuclear protein kinase.

**Table 2.** Effect of NaCl on the bivalent metal ion-dependent nuclear protein kinase activity.

Metal ion (10 mM)	Protein kinase activity (units)	
	Control (- NaCl)	+ NaCl (0.3 M)
None	0	N.D.
MgCl <sub>2</sub>	28	38
CoCl <sub>2</sub>	11	30
MnCl <sub>2</sub>	5	28

The standard assay systems were used except for the specified alterations in relation to NaCl and the bivalent metal ions. N.D.—Not detectable.

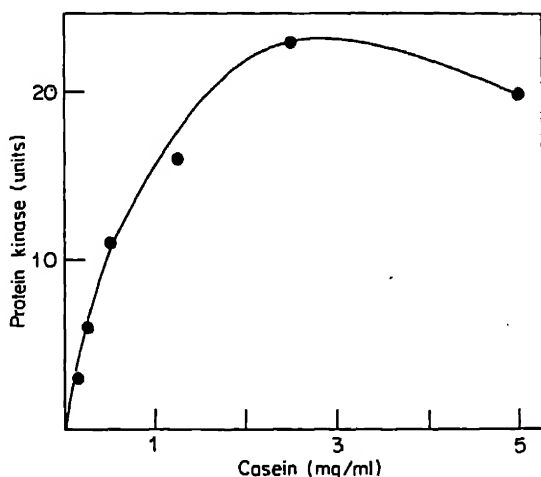
the bivalent metal ion-dependent enzymic activity, the degree of stimulation being dependent on the nature of the activator metal ion. In the presence of Mg<sup>2+</sup>, Co<sup>2+</sup> or Mn<sup>2+</sup>, NaCl stimulated the enzymic activity by approx. 35%, 170% or 460% respectively. Figure 4 shows effect of various concentrations of NaCl on the activity of the nuclear protein kinase. NaCl at a relatively high concentration (0.1–0.3 M) markedly activated (100–200%) the enzyme, the stimulation being maximal at approx. 0.3 M concentration of the salt.

**Figure 4.** Effect of various concentrations of sodium chloride on the activity of nuclear protein kinase.

**Table 3.** Substrate specificity of epididymal nuclear protein kinase.

Protein Substrate	Amount ( $\mu$ g)	Protein kinase activity (units)
Nil	—	0.0
Casein	50	5.0
	250	9.3
Phosvitin	50	0.0
	250	1.1
Calf thymus whole histones	50	0.0
	250	0.0
Protamine	50	0.0
	250	0.0

Standard assay systems were used except for the replacement of 500  $\mu$ g casein by the various substrates as indicated.

**Figure 5.** Effect of various concentrations of casein on the activity of nuclear protein kinase.

### *Substrate specificity*

The ability of the nuclear protein kinase to phosphorylate various model protein substrates was determined (table 3). The enzyme showed a high degree of substrate specificity for casein. Phosvitin was phosphorylated to a smaller extent while there was no detectable phosphorylation of the basic proteins such as histones and protamine. In the absence of exogenous proteins, the enzyme preparation did not show any detectable phosphorylation of endogenous proteins, presumably due to extremely low amount of protein (0.5  $\mu$ g) present in the enzyme preparation added to the assays.

The effect of varying concentrations of casein on the protein kinase activity is shown in figure 5. The enzymic activity was maximal at a casein concentration of approx. 2.5 mg/ml. The apparent  $K_m$  value of the enzyme for casein (calculated on the basis of a direct plot of the data) is 0.6 mg/ml.

### Discussion

The present studies characterize for the first time a protein kinase in epididymal nuclei. The nuclear kinase activity was not due to cytoplasmic contamination of nuclear preparation since the properties of the nuclear enzyme are markedly different from those of the cytosolic cyclic AMP-dependent protein kinases reported earlier (Biswas and Majumder, 1982). For example, unlike the cytosolic kinases, the nuclear enzyme was not dependent on cyclic AMP. Furthermore, the nuclear enzyme had high substrate specificity for the acidic proteins whereas the cytosolic enzymes are specific for the basic proteins (Biswas and Majumder, 1982).

Previous studies had characterized nuclear protein kinases in several tissues other than the epididymis. Single or multiple forms of the nuclear kinase were demonstrated and the profile of the nuclear enzyme differed considerably in various tissues (Takeda *et al.* 1971; Kish and Kleinsmith, 1974; Farron-Furstenthal, 1975; Piras and Piras, 1977; Majumder, 1977; Phillips *et al.*, 1979). Three major types of the nuclear kinases have been characterized: (i) cyclic AMP-dependent protein kinase holoenzyme consisting of a catalytic and a regulatory subunit (Langan, 1973; Walsh and Cooper, 1979), (ii) cyclic AMP-independent protein kinase derived from the cytosolic holoenzyme and (iii) cyclic AMP-independent kinase which is different from the cytosolic catalytic subunit. The epididymal nuclear enzyme is neither holoenzyme nor catalytic subunit that could have been translocated from cytosol (Krall *et al.*, 1978), since the enzymic characteristics of the nuclear kinase are clearly different from those of the epididymal cyclic AMP-dependent cytosolic protein kinase (Biswas and Majumder, 1982).

At present very little is known about the physiological substrates of the epididymal nuclear protein kinase that has been reported here. The observation that the epididymal nuclear kinase has markedly high specificity for the phosphorylation of the acidic protein, casein rather than the nuclear basic proteins (table 3), suggests that the nuclear acidic proteins may serve as physiological substrates for the nuclear protein kinase. The observation that the phosphorylation of nuclear acidic proteins of rat epididymis is under the regulation of testosterone (Kadohama and Turkington, 1974) and that the phosphorylation of the nuclear acidic proteins directly affects the rate of RNA synthesis *in vitro* (Teng *et al.*, 1971; Kamiyama *et al.*, 1972; Shea and Kleinsmith, 1973; Kleinsmith, 1975; Ohtsuki *et al.*, 1980) are consistent with the view that nuclear protein kinase may participate in the regulation of cell differentiation in the epididymis by phosphorylating nuclear proteins.

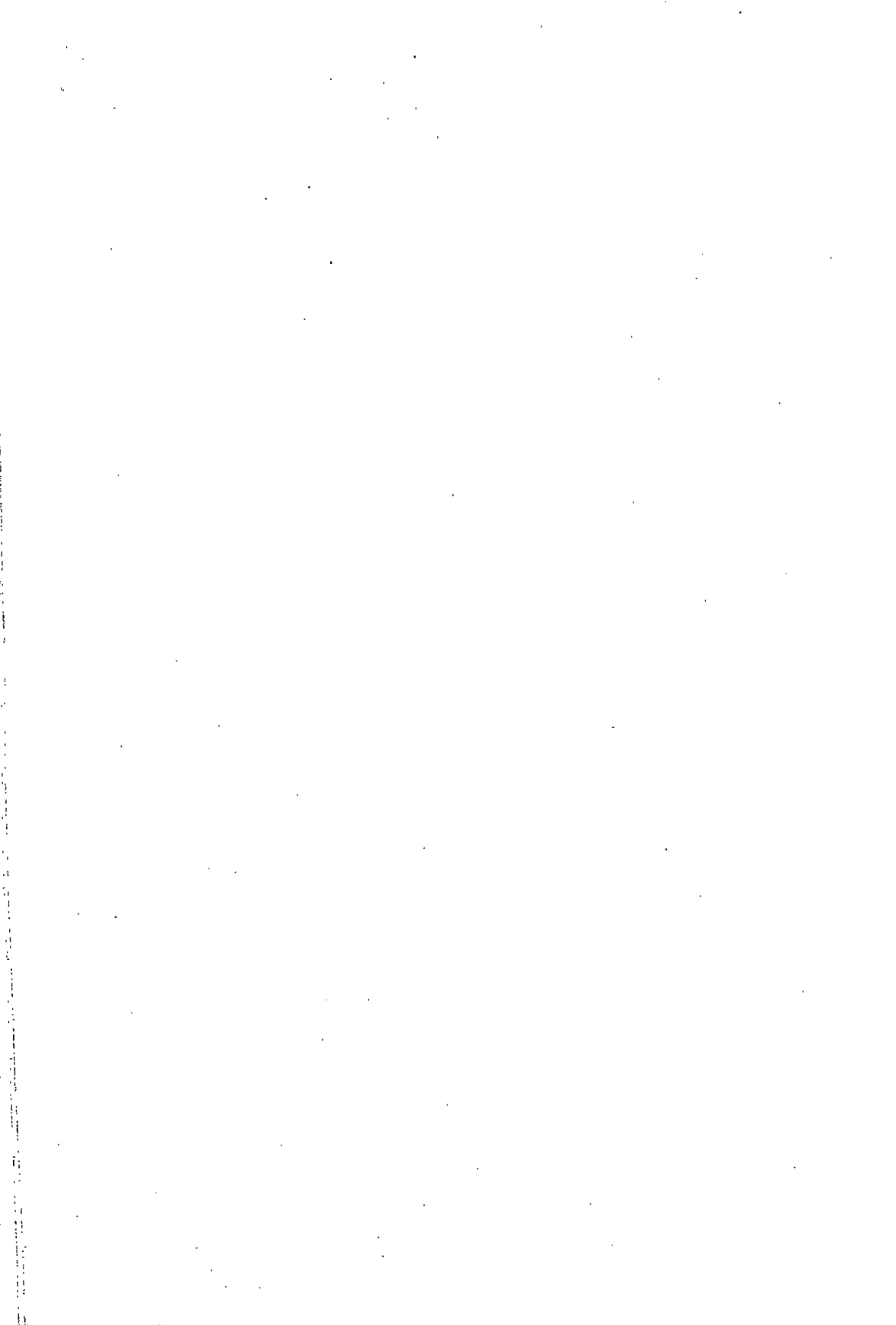
### Acknowledgements

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## The minor anionic form of arylsulphatase B (arylsulphatase Bm) of monkey brain. Purification and phosphoprotein nature

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**Abstract.** The anionic form of arylsulphatase B (arylsulphatase Bm) was purified to apparent homogeneity from monkey brain through steps involving chromatography on diethylaminoethyl-cellulose, Blue-Sepharose, Biogel HTP and finally Biogel P-300 gel filtration. The molecular weight of the purified enzyme as deduced by gel filtration on Biogel P-300 and by sodium dodecylsulphate gel electrophoresis was ~ 30,000. *Escherichia coli* alkaline phosphatase treatment of arylsulphatase Bm resulted in the conversion of upto 84% of the enzyme into a less charged form of enzyme, that could not bind to diethylaminoethyl cellulose. Potassium phosphate an inhibitor of alkaline phosphatase prevented this conversion. Upon acid hydrolysis the purified enzyme yielded approximately 7.0 mol of inorganic phosphate per mol of protein. *Vibrio cholerae* neuraminidase treatment did not alter the charge on arylsulphatase Bm.

**Keywords.** Arylsulphatase Bm; monkey brain; purification; phosphoprotein.

### Introduction

The importance of arylsulphatases A and B stems from their absence in the human genetic disorders metachromatic leukodystrophy and Maroteaux-Lamy syndrome (Austin *et al.*, 1963; Fluaharty *et al.*, 1975). The physiological substrates for arylsulphatases A and B were identified as cerebroside sulphate and UDP-N acetyl galactosamine-4-sulphate respectively. A minor anionic form of arylsulphatase B termed arylsulphatase Bm which is found in the primate brain and which shows many properties of arylsulphatase B is of particular interest for the following reasons (i) It is found in a phosphorylated form only in the primate (human and monkey) brain but not in the brain of other species like rat, chicken or rabbit (J. Mathew and A. S. Balasubramanian, unpublished data), (ii) It is not found in the liver of primates (Stevens *et al.*, 1977; J. Mathew and A. S. Balasubramanian, unpublished data) and (iii) An anionic form of arylsulphatase B termed arylsulphatase B<sub>1</sub> found in transplantable tumours of athymic mice has been reported to be both phosphorylated and sialylated (Gasa *et al.*, 1981).

Arylsulphatase Bm has not been purified and characterized so far. We report here its purification from monkey brain and some of its characteristics which suggest that it is a phosphoprotein.

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Abbreviations used: DEAE, Diethylaminoethyl; SDS, sodium dodecyl sulphate.

## Materials and methods

Blue-Sepharose was prepared by coupling Cibacron Blue F3GA (Ciba Geigy) to Sepharose 6B (Ahmad *et al.*, 1977). Biogel HTP and Biogel P-300 were from BioRad, USA, sodium metaperiodate from J. T. Baker, Phillipsburg, New Jersey, USA, and *Escherichia coli* alkaline phosphatase (Type III) from Sigma Chemicals, St. Louis, Missouri, USA. All other chemicals were obtained as described earlier (Lakshmi and Balasubramanian, 1980).

### *Purification of the enzyme*

Unless otherwise mentioned all operations were done at 0–4°C. Monkey (*Macaca radiata*) brain (70 g) kept frozen at –18°C was thawed and homogenized with 5 volumes of 20 mM Tris-HCl, buffer pH 7.4 in a Waring blender and centrifuged at 12000 *g* for 30 min. The supernatant was dialysed against 100 volumes of the same buffer for 24 h.

### *Diethyl aminoethyl (DEAE)-cellulose ion exchange chromatography*

The dialysed supernatant was loaded on a column packed with precycled DEAE-cellulose (26 × 4.5 cm), washed with 20 mM Tris-HCl buffer, pH 7.4 and eluted with a linear gradient of 0–0.2 M NaCl in the same buffer. Fractions of 25 ml were collected. By this method arylsulphatase B, Bm and A were completely separated from each other (Lakshmi and Balasubramanian, 1980). The active fractions of arylsulphatase Bm were pooled and dialysed against 100 volumes of 20 mM Tris-acetate buffer pH 6.5.

### *Blue Sepharose affinity chromatography*

The dialysed fraction of arylsulphatase Bm from DEAE-cellulose column was loaded on a Blue-Sepharose column (8.2 × 2.8 cm) equilibrated with 20 mM Tris-acetate buffer pH 6.5, washed with the same buffer and with 20 mM Tris-HCl buffer pH 7.4. The enzyme was eluted with 20 mM Tris-HCl buffer pH 7.4/0.25 M NaCl. Fractions of 5 ml were collected and the active fractions were pooled.

### *Hydroxyapatite chromatography*

The pooled fractions of arylsulphatase Bm from the previous step was dialysed against 10 mM Tris-acetate buffer pH 6.5 and loaded on a Biogel HTP column (6.5 × 2.0 cm) equilibrated with the same buffer. The column was initially washed with the loading buffer, then with 0.1 M potassium phosphate buffer pH 7.5 and the enzyme eluted with 0.25 M potassium phosphate buffer pH 7.5. The eluate (60 ml) was dialysed for 2 h against 120 volumes of 20 mM Tris-HCl buffer pH 7.4 with one change. The enzyme concentrated against Aquacide II to 1.5 ml was redialysed exhaustively against the same buffer to remove the phosphate completely.

### *Gel filtration on Biogel P-300*

The concentrated enzyme from the previous step was layered on a Biogel P-300 column (26.5 × 2.1 cm) equilibrated with 20 mM Tris-HCl buffer pH 7.4/0.15 M NaCl. Fractions of 2 ml were collected at a flow rate of 8 ml/h. The active fractions were pooled, concentrated against Aquacide II and dialysed against 20 mM Tris-HCl buffer pH 7.4.

### *Polyacrylamide gel electrophoresis*

The concentrated eluate (12 µg) from Biogel P-300 column was subjected to polyacrylamide gel electrophoresis under non-denaturing conditions in the Davis system (Davis, 1964) as described earlier (Lakshmi and Balasubramanian, 1980). The protein was stained with Coomassie Brilliant Blue G250 according to the method of Holbrook and Leaver (1976).

### *Molecular weight determination by sodium dodecyl sulphate (SDS) gel electrophoresis and gel filtration on Biogel P-300*

Twenty µg of the purified enzyme containing 2% (w/v) SDS with 5% (v/v) 2-mercaptoethanol was boiled for 2 min and subjected to gel electrophoresis according to the method of Laemmli (1970). The electrophoretic mobility was compared against standards of known molecular weights—human IgG (150,000), bovine serum albumin (62,000), ovalbumin (43,000) and soybean trypsin inhibitor (21,000).

The molecular weight of the enzyme was also determined by gel filtration on Biogel P-300 by comparing the  $V_e/V_o$  values with those of the standard marker proteins used in the SDS gel electrophoresis.

### *Treatment with E. coli alkaline phosphatase*

*E. coli* alkaline phosphatase treatment of the Bm enzyme (obtained after the Blue-Sepharose chromatography step) was carried out as follows. The incubation mixture in a final volume of 2.0 ml contained the enzyme protein, 10 mM MgCl<sub>2</sub>, 100 mM Tris-acetate buffer pH 8.0 and *E. coli* alkaline phosphatase (2 units/mg protein). After incubation for 3 h or 5 h at 37°C, the mixture was dialysed against 100 volumes of 20 mM Tris-HCl buffer pH 7.4 with 4 changes and loaded on a DEAE-cellulose column (9.8 cm × 0.8 cm) equilibrated with the same buffer. The column was washed, eluted with 0.2 M NaCl in the buffer and the fractions were assayed using nitro catechol sulphate as substrate. In the case of the purified Bm enzyme, the incubation mixture contained 17.4 µg protein, 1 unit of alkaline phosphatase, 10 mM MgCl<sub>2</sub> and 100 mM Tris-acetate buffer pH 8.0 in a final volume of 1.0 ml. After an incubation period of 1 h, it was dialysed and passed through a DEAE-cellulose column (7.5 cm × 0.8 cm) as before. The fractions were assayed for activity using 4-methyl umbelliferyl sulphate as substrate.

*Treatment with Vibrio cholerae neuraminidase*

The Blue-Sepharose eluate of arylsulphatase Bm (1.36 mg protein) was incubated with *V. cholerae* neuraminidase (0.1 unit) in the presence of 5 mM  $\text{CaCl}_2$  and 100 mM sodium-acetate buffer pH 5.5 for 1 h at 37°C. The incubation mixture after dialysis against 20 mM Tris-HCl buffer pH 7.4 was passed through a DEAE-cellulose column and eluted as described for the phosphatase treatment.

*Treatment with sodium metaperiodate*

The Bm enzyme (DEAE-cellulose fraction, 1.7 mg protein) was concentrated by Aquacide II and dialyzed against 10 mM phosphate buffer, pH 6.0. It was incubated with 10 mM sodium metaperiodate for 2 h at 4°C in the dark. Ethylene glycol (0.1 ml) was added to arrest the reaction and dialysed against 20 mM Tris-HCl buffer pH 7.4. Incubation of the enzyme with periodate for periods longer than 2 h resulted in considerable loss of activity (about 80% loss for 6 h). The mixture was passed through a DEAE-cellulose column and eluted as described for phosphatase treatment.

*Acid hydrolysis of purified arylsulphatase Bm*

The homogeneous arylsulphatase Bm (29  $\mu\text{g}$ ) was hydrolysed with 6 N HCl at 110°C for 8 h in a sealed pyrex hydrolysis tube. After hydrolysis, the sample was dried by repeated evaporation under vacuum over KOH to remove HCl completely and the phosphate liberated was quantitated by the method of Chen *et al.* (1956).

*Enzyme assays*

The incubation mixture for arylsulphatase Bm contained 7.5 mM nitrocatechol sulphate, 0.2 M sodium-acetate buffer, pH 5.5 and the enzyme in a total volume of 0.2 ml. One unit of enzyme activity corresponds to 1  $\mu\text{mol}$  of nitrocatechol released/h (Lakshmi and Balasubramanian, 1980).

In experiments where 4-methyl umbelliferyl sulphate was used as the substrate, the incubation mixture contained 2.5 mM 4-methyl umbelliferyl sulphate, 0.2 M sodium-acetate buffer, pH 5.5 and the enzyme in a total volume of 0.2 ml. After incubation at 37°C for 4 h, the reaction was stopped by the addition of 1.0 ml of 0.4 M glycine-NaOH buffer, pH 10.5 and the fluorescence of 4-methyl umbelliferone was measured in a Hitachi 204-A fluorescence spectrometer (Stevens *et al.*, 1977).

Protein was measured according to Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

**Results and discussion***Purification of arylsulphatase Bm*

Table 1 shows the purification data of a typical batch of arylsulphatase Bm from monkey brain. The enzyme at the final step of gel filtration on Biogel P-300 was 87 fold

Table 1. Purification of arylsulphatase Bm from monkey brain

Purification step	Total units*	Specific activity (units/mg protein)	Purification fold	Recovery (%)
DEAE-cellulose	83	0.94	—	100
Blue-Sepharose	44	7.0	7	53
Biogel-HTP	12	40.0	42	14
Biogel P-300	10	82.37	87	12

\* 1 unit = 1  $\mu$ mol nitrocatechol released/h.

purified with an overall recovery of 11.5% over the DEAE-cellulose fraction. The enzyme moved as a single diffuse band on polyacrylamide gel electrophoresis under non-denaturing conditions and as a sharp band under denaturing conditions in the presence of SDS. The final yield varied with different batches of purification. This was mainly due to the varying recoveries obtained at the step of Blue-Sepharose chromatography. It should also be mentioned that the final purified enzyme was very labile. Storage of the enzyme at 4°C for 24 h resulted in 90% loss of activity. Addition of 2-mercapto-ethanol or bovine serum albumin did not stabilize the enzyme.

#### Molecular weight determination

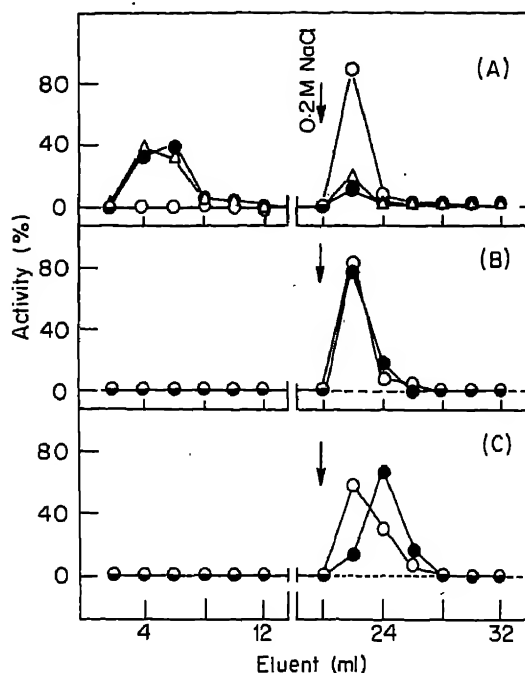
The molecular weight of the purified enzyme by SDS polyacrylamide gel electrophoresis and by gel filtration on Biogel P-300 was found to be 30,000 and 33,000 respectively. Arylsulphatase B (concentrated DEAE-cellulose fraction) was also found to have a molecular weight comparable to that of arylsulphatase Bm on Biogel P-300 gel filtration.

#### Treatment with *E. coli* alkaline phosphatase and *V. cholerae* neuraminidase

The elution profile of the arylsulphatase Bm enzyme (obtained after Blue-Sepharose chromatography) subjected to *E. coli* alkaline phosphatase treatment is shown in figure 1A. About 76% and 84% of the enzyme was converted to a less acidic presumably dephosphorylated form after 3 h and 5 h of incubation respectively. The near absence of any dephosphorylated enzyme in the control experiments upto 5 h of incubation was suggestive of the absence of any endogenous phosphatase activity. When *E. coli* phosphatase treatment was done in the presence of 100 mM potassium phosphate buffer, pH 8.0 (an inhibitor of alkaline phosphatase) there was no conversion of arylsulphatase Bm into the less charged form (not shown in figure).

About 21% of the purified arylsulphatase Bm was converted into an unbound dephosphorylated form on DEAE-cellulose chromatography after treatment with *E. coli* alkaline phosphatase for 1 h. Longer incubation periods was not possible because the purified Bm enzyme lost activity rapidly at 37°C.

Unlike phosphatase, neuraminidase treatment did not result in the conversion of the arylsulphatase Bm into a less acidic form unbound to DEAE-cellulose column (figure



**Figure 1.** Profile of arylsulphatase Bm on DEAE-cellulose column chromatography when subjected to (A) Phosphatase (B) neuraminidase and (C) periodate treatment. (A) Blue Sepharose eluted arylsulphatase Bm incubated with alkaline phosphatase for 3 h ( $\Delta$ ) and 5 h ( $\bullet$ ); without alkaline phosphatase for 5 h ( $\circ$ ). (B) Blue Sepharose eluted arylsulphatase Bm incubated with ( $\bullet$ ) and without ( $\circ$ ) neuraminidase for 1 h. (C) DEAE-cellulose eluted arylsulphatase Bm incubated with ( $\bullet$ ) and without ( $\circ$ ) sodium metaperiodate at  $4^{\circ}\text{C}$  for 2 h. Details of procedure are given under Materials and methods.

1B). Sodium metaperiodate treatment failed to bring about any significant change in the elution profile of the enzyme on DEAE-cellulose (figure 1C).

The dephosphorylated arylsulphatase Bm showed the same molecular weight as the phosphorylated enzyme on Biogel P-300 gel filtration.

#### *Phosphate content of the purified enzyme*

The homogenous enzyme upon acid hydrolysis yielded 7.01 mol of inorganic phosphorus/mol protein. This quantitative estimation of phosphorus confirmed the phosphorylated state of arylsulphatase Bm.

The present studies indicate that arylsulphatase Bm of brain differs from arylsulphatase B<sub>1</sub> of transplantable lung tumours (Gasa *et al.*, 1981) in its molecular weight and in its non-susceptibility to neuraminidase action. Although the phosphorylated nature of purified arylsulphatase Bm has been suggested by the present experiments, the nature of the phosphate residues remains to be determined. Periodate treatment which is known to result in the oxidation of carbohydrate residues failed to



alter the elution profile of arylsulphatase Bm. However this experiment does not completely exclude the presence of sugar phosphate residues (such as mannose-6-phosphate) because the sugar phosphate may still remain attached to the enzyme after periodate treatment. Serine, threonine or tyrosine phosphate residues are known to be present in a number of phosphoproteins. Gasa and Makita (1983) have recently shown the presence of phosphorylated residues on both the protein and carbohydrate moieties in arylsulphatase B<sub>1</sub>. Attempts to label the Bm enzyme with <sup>32</sup>P and analyze the phosphorylated residues are under way now.

### Acknowledgement

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## Genetic analysis of a Minute mutation in the distal region of the second chromosome of *Drosophila melanogaster*

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**Abstract.** Genetic organization of a proximal region of the second chromosome in *Drosophila melanogaster* has been analysed by saturation mutagenesis. Seven alleles were uncovered in this region in addition to the one previously known. Besides this, quite a few mutations were isolated that non-complemented more than one group of lethals and looked very much like deletions of varying extent. Except one, all the lethals complemented M(2)z.

**Keywords.** Genetic fine structure; M(2)-z<sup>B</sup>; lethal isolation; complementation analysis.

### Introduction

Saturation mutagenesis has often been used as a technique to analyse the genome organization of *Drosophila melanogaster*. A number of loci namely white (Judd *et al.*, 1972), Notch (Welshons, 1965), rosy (Hillicker *et al.*, 1980), alcohol dehydrogenase (Woodruff *et al.*, 1979) and several others have been analysed in their detail by this technique, to understand more about their organizational complexity and fine structure. The outcome of these analyses is the identification of several new cistrons which were previously unknown. With a purview to these facts, we started analysing a region at the distal part of second chromosome left arm (2L), spanning the section 24E2-F1 to 25A1(2) on the salivary gland chromosome map of Bridges (Lindsley and Grell, 1968) (figure 1B). The characteristic feature of this region is that it shows Minute phenes in haploid condition. Bridges named it as M(2)-z<sup>B</sup>. Schultz (1929) induced a new Minute mutation — M(2)z which non-complemented this deficiency and which had no apparent chromosomal abnormalities.

Minute mutations in *Drosophila* are a group of dominant mutations at different chromosomal sites or at different sites of the same chromosome, that exhibit a similar phenotypic spectrum *viz.*, small and thin bristles, small body size, prolonged developmental time, somewhat rough eyes and reduced viability and fertility. Schultz (1929) first characterized these mutations phenotypically as a group. Besides these dominant defects, they also act as recessive lethals with death occurring at early embryogenesis or at the first instar (Brehme, 1939; Farnsworth, 1957a, b). Lindsley *et al.* (1972) showed that heterozygosity for small deletions spanning a great part of the *D.*

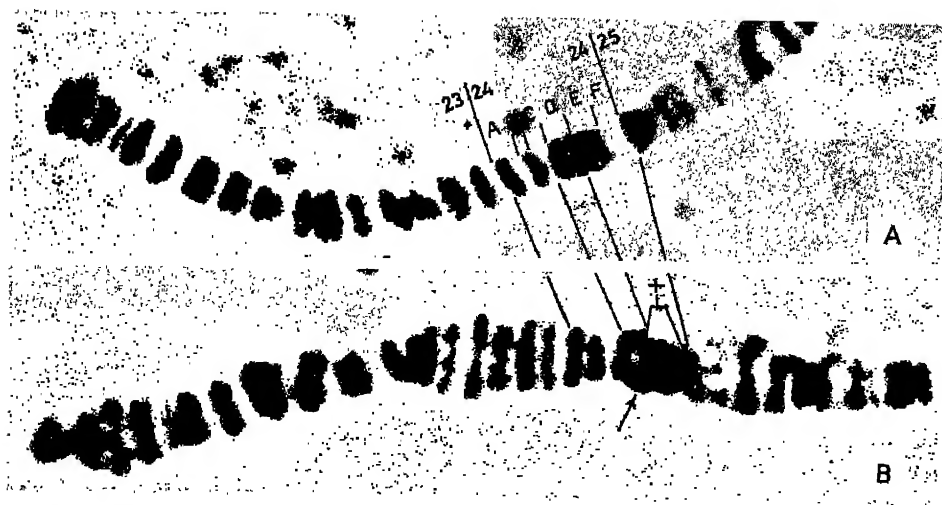


Figure 1. A photograph of the (A)  $M(2)-z^B+$  region of the second chromosome and (B) same region in a deficient heterozygote. '+' Region denotes bands 24E2-F1 to 25A1 in the normal homologue which are not present in the homologue opposite (arrow).

*melanogaster* genome results into minute phenotype *i.e.*, the wild type gene fails to produce a normal product in haploid condition (haplo-insufficient) and 42 such regions were identified by them. Although no workable hypothesis has emerged, a number of propositions have been made to attribute an unitary function for all these loci (Brehme, 1939; Farnsworth, 1965; Ritossa *et al.*, 1966; Sinclair *et al.*, 1981).

Taking advantage of the fact that the minute mutants act as recessive lethals we made an attempt to uncover the possible number of alleles that could be accommodated in the above said chromosomal region *viz.*,  $M(2)-z^B$ . Our data shows that deficiency  $M(2)-z^B$  contains seven complementation groups (cistrons) in addition to the one *i.e.*,  $M(2)z$ , already described by Schultz (1929).

### Materials and methods

All stocks were maintained and crosses were performed on standard cornmeal—sugar—*Drosophila* medium at  $22 \pm 1^\circ\text{C}$  temperature.

### Screening protocol to generate alleles of $M(2)-z^B$

A stock isogenic for chromosome 2 and homozygous for three recessive markers black (*b*, 2:48.5), purple (*pr*, 2:54.5) and cinnabar (*cn*, 2:57.5) was derived. Freshly eclosed males (48 h of eclosion) were fed with ethyl methane sulphonate at a concentration of 0.025 M in 1% sucrose solution for 24 h (Lewis and Bacher, 1968) or X-irradiated (3000–4000 R) by Picker's X-ray machine (110 kv, 4 mA; 720 R/min). The treated males were then transferred to fresh food bottles and allowed to recover for 24 h.

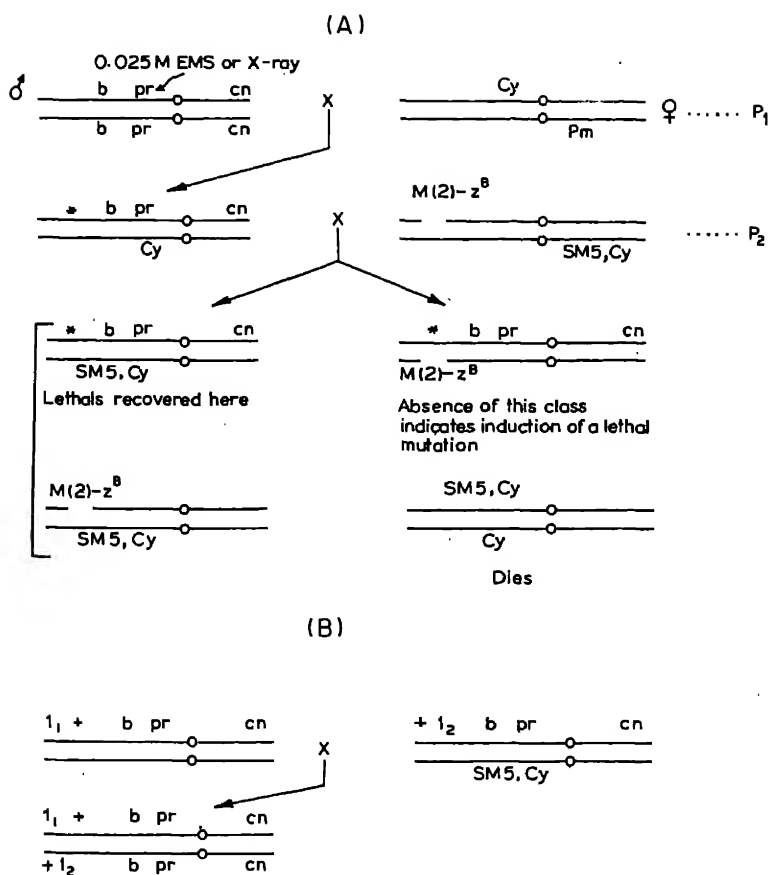


Figure 2. Schematic representation of the protocol used for (A) recovering lethal mutations against Df M(2)-z<sup>B</sup> and (B) for complementation analysis.

These males were then allowed to mate *en masse* to In(2LR)Cy/In(2LR)Pm virgin females for three days after which they are discarded. The F<sub>1</sub> male progeny, heterozygous for treated paternal chromosome, *b pr cn*/In (2LR)Cy, were then pair mated with DfM(2)-z<sup>B</sup>/SM5, Cy virgin females. In the F<sub>2</sub>, a lethal allele for DfM(2)-z<sup>B</sup> was evident from the absence of straight wing flies with minute bristles. The lethal chromosome was recovered and maintained in the *b pr cn*/SM5, Cy heterozygotes. Screening protocol is outlined in figure 2A.

#### Inter se complementation analysis

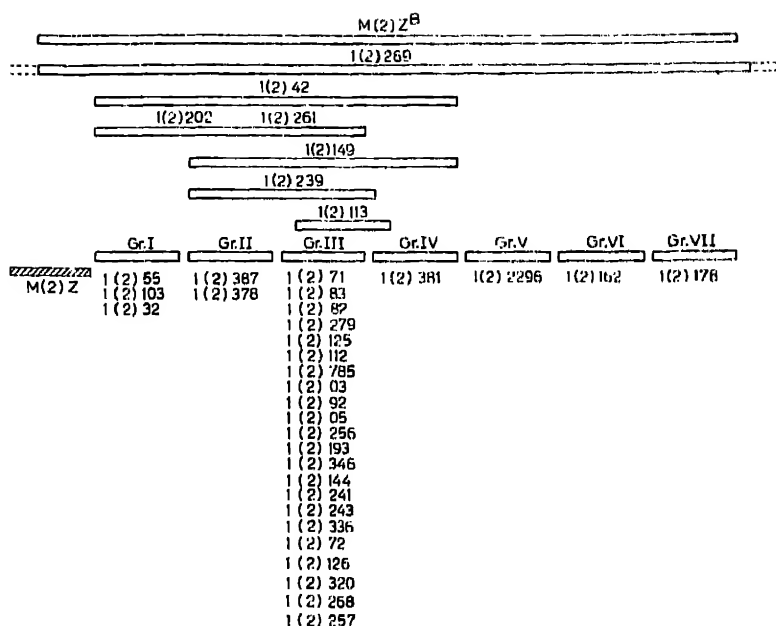
Since all the lethals were recovered in a chromosome marked with *b pr cn*, the recovery of a *b pr cn* progeny from a cross between two lethals, indicated complementation (figure 2B). For this purpose, all lethals were mated *inter se* (5 males: 5 females) and then we looked for homozygous *b pr cn* progenies. Though recovery of the wild type class is

Table 1. The progeny class and the fly numbers recovered for each of the lethals isolated.

Cross	Mutagen	Progeny classes		
		<i>b pr cn/Cy</i>	<i>b pr cn/M(2)-z<sup>B</sup></i>	<i>M(2)-z<sup>B</sup>/Cy</i>
1(2)71 × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	24	0	21
1(2)55 × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	26	0	37
1(2)239/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	44	0	39
1(2)268/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	30	0	21
1(2)103/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	25	0	21
1(2)149/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	38	0	30
1(2)83/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	34	0	36
1(2)82/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	56	0	50
1(2)279/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	36	0	22
1(2)125/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	34	0	33
1(2)112/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	45	0	39
1(2)113/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	38	0	44
1(2)92/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	55	0	49
1(2)126/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	39	0	36
1(2)320/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	35	0	29
1(2)243/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	41	0	38
1(2)05/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	40	0	34
1(2)387/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	70	0	62
1(2)32/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	44	0	38
1(2)42/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	32	0	29
1(2)378/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	47	0	41
1(2)202/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	71	0	65
1(2)256/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	81	0	72
1(2)193/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	38	0	32
1(2)261/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	35	0	32
1(2)785/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	50	0	41
1(2)257/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	57	0	49
1(2)03/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	86	0	81
1(2)346/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	36	0	32
1(2)381/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	44	0	41
1(2)144/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	23	0	21
1(2)241/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	68	0	63
1(2)336/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	33	0	28
1(2)72/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	48	0	40
1(2)2296/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	45	0	40
1(2)269/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	X-ray	—127 Minute flies recovered—		
1(2)162/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	56	0	61
1(2)178/ <i>Cy</i> × <i>M(2)-z<sup>B</sup>/Cy</i>	EMS	65	0	72

EMS, Ethyl methane sulphonate.

an indication of complementation, the extent of survivability may vary within the non-complementing lethals. We are now doing developmental analysis of these mutations and hopefully would be able to estimate the actual span of these mutations.



**Figure 3.** Fine structure map of the alleles isolated against Df M(2)-z<sup>B</sup>. Since we are engaged in finding the map position of the complementation groups with respect to each other, the present linear arrangement of the group is arbitrary.

### Allelism test with M(2)-z

All the lethals isolated in the deficient region were tested for allelism with M(2)z. Following the same criterion as in Df M(2)-z<sup>B</sup>, each lethal allele was crossed with M(2)z/SM5 virgins and progenies were scored for the presence or absence of straight winged Minute flies.

### Results and discussion

A total of 6,675 chromosomes were tested from which 38 recessive lethals were recovered. No recessive visible mutation could be found. Table 1 shows that complete absence of a particular class *i.e.*, with straight wing and normal bristles in the culture are regarded as lethals. To avoid confusion, we have not considered any semi-lethals which shows greater viability than their lethal sibs, though a number of such have been recovered during saturation.

*Inter se* complementation analysis resolved these 38 lethal mutations into seven complementation groups with some overlappings (figure 3). We, therefore, were able to identify at least seven vital or essential loci as described by Hochman (1971), that could be mutated to lethal state. As we are still engaged in obtaining suitable overlapping deletions and duplication spanning this region, separation of the adjacent complemen-

**Table 2.** Results of *inter se* complementation test between one of the representative of each group. Figures represent proportion of non-Cy vs Cy winged flies.

	1(2)55	1(2)162	1(2)178	1(2)113	1(2)378	1(2)381	1(2)2296
1(2)55	0/0	72/154	68/145	100/201	44/90	52/114	58/112
1(2)162			54/96	62/114	46/108	60/131	42/89
1(2)178				74/138	58/124	62/130	51/112
1(2)113					58/114	0/94	48/108
1(2)378						53/110	66/130
1(2)381							64/120
1(2)2296							0/0

tation groups is yet to be completed. Two of these lethals namely, 1(2)202 and 1(2)261 show non-complementation with Group I, II and III. Similarly, 1(2)149 shows non-complementation with Group II, III and IV and 1(2)239 is non-complementing Group II and III. Incidentally, these lethals are all X-ray induced. Our attempt to detect any cytological deletion for these lethals have failed. Lim and Snyder (1974) have shown the inability of mono-functional alkylating agents to produce deletion mutations. Therefore, these mutations, that non-complement more than one group are supposed to be cytologically non-detectable deletions, while the rests are regarded as single site lesions. One lethal namely, 1(2)269 has been found to non-complement all the seven groups including M(2)z. It also shows Minute phenotype and therefore, may be regarded as deletion either equal or bigger than M(2)-z<sup>B</sup>.

Of the 38 mutations recovered, 23 are included in Group III and this is regarded as a mutational hot spot. Table 2 is a coinicise form of the complementation matrix. From each group a representative mutation is chosen and its genetic behaviour is shown with representative of the other groups. Group III representative 1(2)113, has been found to non-complement Group IV, whereas several other lethals belonging to this group, complement Group IV. Here again we regard 1(2)113 as a small scale deletion. Shanon *et al.* (1972) in their study of 3A-3C region of X-chromosome identified 13 complementation groups (cistrons) by analysing the developmental pattern of an array of mutants belonging to each complementation group. Detail work of the developmental pattern of the lethal is in progress and are indicative of an interesting correlation of developmental timings with the complementation groups.

The situation of M(2)z and M(2)-z<sup>B</sup> shows a very good parallelism with the two, 4th chromosome mutation M(4)<sup>57g</sup> and M(4)<sup>63a</sup> (Hochman, 1971). Taking viability as the only criterion, allelism test with M(2)z (data presented in table 3) shows that none of the lethals that non-complements M(2)-z<sup>B</sup>, non-complements M(2)z locus. This led us to assume that M(2)z occupy either border of the deficiency and it may act in a trans-dominant fashion to kill the M(2)z/M(2)-z<sup>B</sup> heterozygote. Recently Broderick and Roberts (1982), with the help of overlapping duplications as well as single band duplications, assigned M(2)z in the polytene chromosome band 25A1(2). This correspond well with the preliminary data of our recombination analysis, where none of the alleles can be placed beyond M(2)z.



Table 3. Showing the allelism test of the 38 lethals with M(2)z

Cross	Progeny classes		
	1 b pr cn/SM5	M(2)z/1 b pr cn	M(2)z/Cy
1(2)71/Cy × M(2)z/SM5	11	14	21
1(2)55/Cy × M(2)z/SM5	23	16	18
1(2)239/Cy × M(2)z/SM5	25	14	11
1(2)268/Cy × M(2)z/SM5	15	11	16
1(2)103/Cy × M(2)z/SM5	27	20	21
1(2)149/Cy × M(2)z/SM5	24	10	21
1(2)83/Cy × M(2)z/SM5	27	18	22
1(2)82/Cy × M(2)z/SM5	15	19	23
1(2)279/Cy × M(2)z/SM5	24	14	27
1(2)125/Cy × M(2)z/SM5	23	26	19
1(2)112/Cy × M(2)z/SM5	17	13	23
1(2)113/Cy × M(2)z/SM5	14	18	22
1(2)92/Cy × M(2)z/SM5	19	15	24
1(2)126/Cy × M(2)z/SM5	23	22	20
1(2)320/Cy × M(2)z/SM5	20	18	26
1(2)243/Cy × M(2)z/SM5	19	14	11
1(2)05/Cy × M(2)z/SM5	19	18	19
1(2)387/Cy × M(2)z/SM5	26	22	23
1(2)32/Cy × M(2)z/SM5	13	15	10
1(2)42/Cy × M(2)z/SM5	17	14	21
1(2)378/Cy × M(2)z/SM5	19	17	20
1(2)202/Cy × M(2)z/SM5	20	18	22
1(2)256/Cy × M(2)z/SM5	18	18	25
1(2)193/Cy × M(2)z/SM5	27	22	16
1(2)261/Cy × M(2)z/SM5	21	19	25
1(2)785/Cy × M(2)z/SM5	24	23	30
1(2)257/Cy × M(2)z/SM5	25	20	18
1(2)03/Cy × M(2)z/SM5	24	20	20
1(2)346/Cy × M(2)z/SM5	18	16	14
1(2)381/Cy × M(2)z/SM5	25	20	23
1(2)144/Cy × M(2)z/SM5	18	17	18
1(2)241/Cy × M(2)z/SM5	20	16	18
1(2)336/Cy × M(2)z/SM5	25	19	22
1(2)72/Cy × M(2)z/SM5	16	18	21
1(2)269/Cy × M(2)z/SM5	All minute flies observed		
1(2)2296/Cy × M(2)z/SM5	24	26	20
1(2)162/Cy × M(2)z/SM5	22	21	25
1(2)178/Cy × M(2)z/SM5	28	21	19

Based on the number of cistron and amount of DNA in each chromomere, Judd *et al.* (1972) regarded each chromomere to consist mainly of regulatory element and the actual coding part (structural element) is very small. Therefore, mutation in any region of the chromomere may make the chromomere non-functional and therefore any two mutations in a single chromomere would non-complement in trans. Woodruff and Ashburner (1979) however, regarded lethality as a mutation in the control region of the

chromomere only. Converging these two ideas we may validly claim that all the lethals belonging to one complementation group, represent mutation in a single chromomere regardless of whether they are in the structural or regulatory region.

Huang and Baker (1976) claimed that the genetic organization of Minutes do not support the contention that they are redundant structural loci for t'RNAs. We too did not find any characteristic of this region to call it redundant, though we are still unable to explain the unusual behaviour of M(2)z, which gives indications of potentially functional redundant structural loci like the ribosomal genes, histone genes and others usually not allowing any lethal mutation within it.

In conclusion, it may be said that the two Minutes, viz. M(2)-z<sup>h</sup> and M(2)z, though non-complementing are complex loci; since all the lethals except 1(2)269, that non-complement M(2)-z<sup>h</sup>, complements M(2)z. Perhaps by a very restricted type of recessive lethality or by a transdominant control, it could non-complement M(2)-z<sup>h</sup> or it may occupy a terminal position of the M(2)-z<sup>h</sup>, which could not be saturated with lethal mutations. The eight cistrons [inclusive of M(2)z] and five overlapping groups within them roughly correspond to the 10 bands which we could perceive with our best approximation. We are, however, still hopeful in finding out one or two more complementation groups and such work is in good progress.

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## **Comparative study of fatty acid composition in human and monkey aorta**

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**Abstract.** The fatty acid composition of thoracic and abdominal aortic intima and media of normal human subjects and rhesus monkeys has been studied. Significantly higher values of unsaturated fatty acids as compared to saturated fatty acids have been noted in the intima of monkey as compared to man. The fatty acid profile of the aortic wall in these two species has provided a probable biochemical basis for the lesser incidence of atherosclerosis in macaques.

**Keywords.** Fatty acids; human and monkey aorta.

### **Introduction**

In our previous study significant differences were observed in the lipid constituents of the aortic wall of man and monkeys (Dahiya *et al.*, 1983). The important differences pertained to the content of total lipids, total cholesterol and its fractions and phospholipids which were significantly more in human aorta than in monkeys. Further, it was observed that the content of neutral lipids was much higher in the intima than in media of both species. Although total phospholipids were not significantly different between intima and media, phosphatidylcholine and phosphatidylethanolamine showed significant alterations. As there was much less atherosclerosis in monkeys as compared to man (Chakravarti and Kukreja, 1981) the above findings with respect to lipid constituents of aorta provide a possible biochemical basis, for this difference. It was, therefore, thought worthwhile to study in detail the fatty acid composition of different lipid constituents of the aortic media and intima which could throw further light on this aspect.

### **Materials and methods**

#### *Collection of aorta samples and separation of intima and media*

As reported earlier (Dahiya *et al.*, 1983), samples of aorta were collected from 18 normal healthy male rhesus monkeys and from 11 young male human subjects who had died in

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street accidents. The upper 2/3rd of the thoracic and abdominal aorta which were sampled for study of lipid profile and intima-media were separated as reported earlier (Smith *et al.*, 1967).

#### *Extraction and separation of lipids and preparation of methyl ester*

The lipids were extracted by the procedure of Folch *et al.* (1957). The different components of neutral lipids like triglycerides, esterified cholesterol and free fatty acids along with total phospholipids were separated by thin layer chromatography using different solvent systems. The different lipids were converted to methyl esters by dissolving them in 1 ml of benzene and 2 ml of 0.5 N sodium methoxide in anhydrous methanol (Christie, 1973); incubated at 50°C for 10 min and 0.1 ml of acetic acid then added. A mixture of 5 ml of water and 5 ml of hexane was added twice, the hexane layer was separated, dried over anhydrous sodium sulphate containing 10% solid sodium bicarbonate and then filtered. The solvent was evaporated in a nitrogen atmosphere. The resulting methyl ester was dissolved in 1 ml of benzene and used in gas liquid chromatography for determination of fatty acid composition.

#### *Gas liquid chromatography*

The methyl esters of fatty acids of different lipid constituents were analysed by gas liquid chromatography using a Pye Unicam 104 instrument fitted with a flame ionization detector under isothermal condition (180°C). The column (6' × 4 mm) contained 10% diethylene glycol succinate on diatomic C-AW (100–200 mesh). Nitrogen gas (40 ml/min) was used as a carrier gas. Fatty acids were identified by comparing their retention times with those of standard fatty acid methyl esters.

### **Results**

Table 1 shows the fatty acid composition of monkey thoracic aorta. The main fatty acids in the phospholipids were stearic, palmitic, oleic, linoleic and arachidonic acid in both intima and media. There was a significant increase in oleic and linoleic acid, and decrease in stearic and palmitic acid of phospholipids in the media as compared to intima. The total saturated fatty acids of phospholipids were significantly decreased while unsaturated fatty acids were higher in media as compared to intima. The main fatty acids of esterified cholesterol were palmitic, palmitoleic, oleic and linoleic acid. There was a significant decrease in palmitic acid in media as compared to intima of monkey thoracic aorta; other fatty acids of esterified cholesterol remained unaltered. The fatty acid content of triglycerides was mainly constituted of palmitic, palmitoleic, oleic and linoleic acid. There was significant decrease in the content of myristic, palmitic and stearic acid and increase in palmitoleic, linoleic and arachidonic acid in media as compared to intima. Total free fatty acid of intima and media of monkey thoracic aorta mainly comprised of palmitic, palmitoleic, stearic, oleic and linoleic acid. There was significant decrease in palmitic and stearic acid content in media as compared to intima. Total saturated fatty acids of esterified cholesterol, triglycerides and free fatty acids

Table 1. Fatty acid composition of monkey thoracic aorta.

	Intima				Media			
	PL	ECH	TG	FFA	PL	ECH	TG	FFA
C <sub>12:0</sub> Lauric acid	1.8±0.5	1.1±0.05	1.7±0.06	1.2±0.27	1.2±0.07	0.9±0.08	1.5±0.09	0.9±0.29
C <sub>14:0</sub> Myristic acid	3.2±0.7	1.4±0.07	5.1±1.2	4.1±1.2	2.1±0.3	1.2±0.1	2.5±0.7**	3.8±1.2
C <sub>16:0</sub> Palmitic acid	22.0±4.6	17.3±4.2	18.6±2.3	16.5±3.2	17.7±4.2*	11.3±2.1*	10.8±2.3***	10.7±2.8*
C <sub>16:1</sub> Palmitoleic acid	4.8±0.9	14.2±4.3	14.7±2.3	5.7±1.2	6.2±1.2	11.9±3.2	21.7±4.2*	8.7±2.9
C <sub>18:0</sub> Stearic acid	19.7±4.2	6.7±1.2	10.5±1.8	16.4±2.3	10.4±1.8**	7.2±1.7	5.6±1.2**	9.7±1.8**
C <sub>18:1</sub> Oleic acid	14.1±2.7	31.5±8.7	36.5±8.2	31.2±5.6	22.4±4.5*	33.5±8.5	34.3±8.5	35.2±9.2
C <sub>18:2</sub> Linoleic acid	6.2±1.2	24.1±4.6	10.2±2.1	20.5±4.2	12.1±2.3***	29.4±3.8	20.2±6.8*	25.5±6.7
C <sub>20:4</sub> Arachidonic acid	27.3±3.2	3.1±0.7	1.8±0.07	3.5±0.7	26.8±3.7	3.7±0.4	2.9±0.7*	4.7±1.3
C <sub>22:0</sub> Behenic acid	0.9±0.1	0.6±0.05	0.9±0.26	0.9±0.07	0.8±0.09	0.9±0.08	0.5±0.28	0.8±0.09
Saturated	47.3±4.5	34.7±4.7	36.6±2.9	38.9±3.2	32.1±3.1***	22.1±2.4**	20.7±2.5***	25.4±2.1***
Unsaturated	52.7±4.8	65.3±7.1	63.4±5.9	61.1±4.7	67.9±4.3***	77.9±6.8*	79.3±6.9*	74.6±6.8*

Values are Mean ± SE of 5 observations. \*P &lt; 0.05; \*\*P &lt; 0.01; \*\*\*P &lt; 0.001.

PL, Phospholipids; ECH, esterified cholesterol; TG, triglycerides; FFA, free fatty acid.

were significantly less and total unsaturated fatty acids were more in media as compared to that of intima of thoracic aorta of monkeys.

Intimal and medial fatty acid composition of monkey abdominal aorta is shown in table 2. The major phospholipid fatty acids were palmitic, stearic, oleic, linoleic and arachidonic acid. There was a decrease in phospholipid palmitic and stearic acid, while an increase in linoleic acid in media as compared to that of intima in monkey abdominal aorta was observed. Esterified cholesterol fatty acid profile showed the palmitic, palmitoleic, stearic, oleic and linoleic acids. Palmitic and stearic acids were significantly less while palmitoleic, oleic and linoleic acids were more in media as compared to intima. The triglycerides mainly contained palmitic, palmitoleic, stearic, oleic and linoleic acid. There were significantly lower values of palmitic and stearic acids while palmitoleic and linoleic acids in media were more as compared to intima. The main constituents of total free fatty acids were palmitic, palmitoleic, stearic, oleic and linoleic acid. There was a significant increase in palmitoleic acid and decrease in stearic acid in media as compared to intima. Total saturated fatty acids of phospholipids, esterified cholesterol, triglycerides and free fatty acids were significantly lower than total unsaturated fatty acids in media as compared to intima of monkey abdominal aorta. A comparison of the fatty acid composition of thoracic and abdominal segments of monkey aorta, revealed that there was more saturated fatty acids in abdominal aorta as compared to thoracic aorta, except for phospholipid fatty acids which were not changed. This shows that increased level of saturated fatty acids and decreased level of unsaturated fatty acids in abdominal aorta might be one of the factors responsible for more atherosclerotic lesions in abdominal segment as compared to thoracic aorta of the same species.

Table 3 shows fatty acid composition of human thoracic aorta. The main phospholipid fatty acids in human thoracic aorta were palmitic, stearic, oleic, linoleic and arachidonic acids. In thoracic aorta, a significant increase in linoleic acid in media was obtained when compared to that in intima. The esterified cholesterol fatty acids, mainly comprised of palmitic, palmitoleic, oleic and linoleic acids. There was a significant increase in palmitoleic acid and decrease in palmitic acid in media as compared to that of intima of human thoracic aorta. The main fatty acid components of triglycerides were palmitic, stearic, oleic and linoleic acids. Stearic and behenic acids were significantly less, while linoleic acid was higher in media as compared to intima. The fatty acid constituents of free fatty acid component were mainly palmitic, stearic, oleic, and linoleic acids. There was a significant decrease in stearic and behenic acid while increase in palmitoleic and oleic acid in media as compared to intima of human thoracic aorta. Total saturated fatty acids of phospholipids, esterified cholesterol, triglycerides and free fatty acids were significantly higher and total unsaturated fatty acids were lower in intima as compared to media of human thoracic aorta.

If fatty acid constituents of human and monkey thoracic aorta were compared, then it was observed that the saturated fatty acid content in human thoracic aorta was higher than that of monkey. The unsaturated fatty acids were more in monkey thoracic aorta.

The fatty acid composition of human abdominal aorta is given in table 4. The main phospholipid fatty acids in intima and media of abdominal aorta were palmitic, stearic, oleic and linoleic acids. The stearic acid was significantly less while oleic, linoleic and arachidonic acids were significantly higher in media as compared to intima. The



Table 2. Fatty acid composition of monkey abdominal aorta

	Intima					Media				
	PL†	ECH†	TG†	FFA†	PL†	ECH†	TG†	FFA†		
C <sub>12:0</sub> Lauric acid	1.6±0.07	1.2±0.09	1.9±0.07	1.4±0.07	1.4±0.08	0.08±0.06	1.6±0.07	0.8±0.09		
C <sub>14:0</sub> Myristic acid	4.7±1.3	4.5±0.8	6.7±1.3	5.1±1.1	3.3±0.6	4.7±0.12	3.2±0.5	4.2±0.8		
C <sub>16:0</sub> Palmitic acid	24.1±3.8	26.6±4.8	27.2±5.6	16.5±5.1	16.5±2.7*	11.9±2.1***	11.8±2.3***	12.7±3.2		
C <sub>16:1</sub> Palmitoleic acid	4.6±0.8	8.8±1.2	7.8±1.5	6.3±1.2	6.1±1.2	12.2±2.8*	11.6±3.6*	9.5±1.7*		
C <sub>18:0</sub> Stearic acid	16.4±5.2	12.9±3.2	18.8±3.4	21.5±4.2	10.5±2.1*	7.5±1.3*	7.2±1.2***	14.3±2.7*		
C <sub>18:1</sub> Oleic acid	13.1±3.4	22.5±3.8	26.5±5.7	27.2±6.8	8.2±4.1	31.5±4.2*	33.2±8.9	29.7±8.7		
C <sub>18:2</sub> Linoleic acid	7.5±1.7	18.9±2.8	9.6±2.3	18.5±4.7	12.4±2.7*	27.3±3.9*	28.1±6.2***	25.3±6.4		
C <sub>20:4</sub> Arachidonic acid	27.4±2.8	3.8±0.9	1.2±0.07	2.7±0.5	30.9±2.2	3.7±0.7	2.8±0.7	2.9±0.8		
C <sub>22:0</sub> Behenic acid	0.6±0.08	0.8±0.06	0.3±0.03	0.8±0.05	0.7±0.06	0.4±0.05	0.5±0.06	0.6±0.03		
Saturated	46.8±5.3	48.2±6.3	53.6±5.2	44.9±3.2	33.4±4.6**	26.4±3.1***	23.3±2.5***	32.4±2.2***		
Unsaturated	53.2±4.9	51.8±5.7	46.4±3.9	55.1±5.4	66.6±4.9**	73.6±4.9***	76.7±6.5***	67.6±6.1*		

Values are Mean ± SE of 5 observations. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

† For expansion please see foot note in table 1.

Table 3. Fatty acid composition of human thoracic aorta

	Intima				Media			
	PL†	ECH†	TG†	FFA†	PL†	ECH†	TG†	FFA†
C <sub>12:0</sub>	21±03	09±01	12±02	11±02	18±02	06±009	13±02	12±01
C <sub>14:0</sub>	42±12	24±12	42±12	39±09	29±07	18±03	24±06*	37±11
C <sub>16:0</sub>	268±47	215±57	267±73	205±43	228±37	138±28*	268±65	204±52
C <sub>16:1</sub>	33±08	98±23	64±18	44±12	26±07	158±22*	67±17	69±012**
C <sub>18:0</sub>	225±53	65±21	147±32	199±63	206±62	46±13	76±21*	116±27*
C <sub>18:1</sub>	168±42	305±72	339±87	241±65	204±35	329±67	394±69	337±56*
C <sub>18:2</sub>	129±37	251±45	98±32	193±47	187±31*	249±48	163±23*	182±21
C <sub>20:4</sub>	102±28	29±08	19±08	62±21	95±420	48±120	08±004	38±12
C <sub>22:0</sub>	12±03	04±01	12±03	06±01	07±01	08±01	03±005***	03±006***
Saturated	578±65	347±47	475±34	454±32	458±49*	296±48	379±25**	369±29*
Unsaturated	422±49	653±71	525±54	546±49	542±47*	714±85	621±61*	631±61

Values are Mean ± SE of 5 observations. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

† For expansion please see foot note in table 1.

Table 4. Fatty acid composition of human abdominal aorta

	Intima				Media			
	PL†	ECH†	TG†	FFA†	PL†	ECH†	TG†	FFA†
C <sub>12:0</sub>	1.3 ± 0.2	1.3 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	0.7 ± 0.05	1.1 ± 0.06	1.3 ± 0.03	1.3 ± 0.05
C <sub>14:0</sub>	4.4 ± 1.3	3.2 ± 0.9	5.8 ± 1.5	3.8 ± 0.9	2.8 ± 1.2	1.8 ± 0.05	2.6 ± 0.3**	3.5 ± 0.7
C <sub>16:0</sub>	28.7 ± 4.2	22.7 ± 5.2	30.8 ± 4.8	31.4 ± 7.2	23.5 ± 3.5	14.3 ± 3.8*	22.4 ± 7.2	20.3 ± 4.7*
C <sub>16:1</sub>	3.4 ± 0.3	8.9 ± 1.3	4.7 ± 1.2	4.2 ± 0.9	2.0 ± 0.7	12.5 ± 2.8	7.3 ± 2.1*	6.3 ± 0.2
C <sub>18:0</sub>	33.2 ± 4.8	16.3 ± 2.3	24.3 ± 5.8	27.1 ± 4.2	24.7 ± 3.2*	10.2 ± 1.7**	8.2 ± 2.3***	12.5 ± 1.7***
C <sub>18:1</sub>	12.5 ± 2.1	21.4 ± 3.8	22.7 ± 4.1	14.4 ± 2.3	18.7 ± 2.3*	27.4 ± 4.2*	38.4 ± 7.8*	35.8 ± 6.3***
C <sub>18:2</sub>	10.2 ± 1.2	22.7 ± 4.2	8.1 ± 1.2	12.7 ± 1.9	15.7 ± 3.1*	27.8 ± 6.3*	17.5 ± 3.1***	18.2 ± 2.5*
C <sub>20:4</sub>	5.1 ± 1.7	2.8 ± 0.3	1.3 ± 0.5	4.3 ± 0.8	10.2 ± 2.3*	4.3 ± 1.2*	1.5 ± 0.3	2.1 ± 0.5**
C <sub>22:0</sub>	1.2 ± 0.2	0.7 ± 0.05	0.9 ± 0.06	0.9 ± 0.07	1.7 ± 0.07	0.6 ± 0.05	0.8 ± 0.05	0.5 ± 0.06
Saturated	63.8 ± 6.2	45.2 ± 4.3	62.9 ± 6.2	63.3 ± 6.4	52.8 ± 3.9*	32.7 ± 3.9**	34.3 ± 2.7***	37.7 ± 2.9***
Unsaturated	36.2 ± 3.1	54.8 ± 5.4	37.1 ± 3.2	36.7 ± 2.9	45.2 ± 3.4**	67.3 ± 6.4**	65.7 ± 6.6***	62.3 ± 6.2***

Values are Mean ± SE of 5 observations. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

† For expansion please see foot note in table 1.

esterified cholesterol fatty acids were mainly palmitic, palmitoleic, stearic, oleic and linoleic acids. There was a significant decrease in palmitic and stearic acids while an increase in oleic, palmitoleic and arachidonic acids in media as compared to intima of human abdominal aorta. The triglyceride fatty acids mainly constitute palmitic, palmitoleic, stearic, oleic and linoleic acids. There was lower value of myristic and stearic acids with increase in palmitoleic, oleic and linoleic acids in media as compared to intima. Similarly, fatty acid constituents of free fatty acid compounds were mainly palmitic, stearic, oleic and linoleic acids. There was significantly higher value of palmitic, stearic acid in intima as compared to media of human abdominal aorta. Total saturated fatty acids of phospholipids, triglycerides, esterified cholesterol and free fatty acids were significantly lesser than that of unsaturated fatty acids in media as compared to intima of human abdominal aorta. If we compare human abdominal aorta with monkey abdominal aorta, then there is more saturated fatty acids and less unsaturated fatty acids in human abdominal aorta as compared to that of monkey.

## Discussion

It is evident from this study that total saturated fatty acid (mainly stearic and palmitic) of phospholipids, esterified cholesterol, triglycerides and free fatty acids are less while unsaturated fatty acids (mainly linoleic, oleic and palmitoleic) are more in the media as compared to intima of monkey thoracic aorta. Similar type of results were obtained in monkey abdominal aorta also. It is well known that saturated cholesterol esters are preferentially deposited in the aortic wall (Sinclair, 1956). Hence the presence of higher content of saturated fatty acids in intima than in media may be one of the factors causing intimal lipidosi. Some workers have emphasised the relationship between polyunsaturated fatty acids and the genesis of either spontaneous or experimental atherosclerotic lesions (Kritchevsky *et al.*, 1954, 1956). It was reported that in humans (Swell *et al.*, 1960a,b) as well as in cholesterol fed rabbits (Evrard *et al.*, 1961; Zilversmit *et al.*, 1961) and cockerels (Blomstrand and Christensen, 1961) that cholesterol ester fraction of the aortic fatty plaque contained a higher percentage of oleic acid and lower percentage of linoleic acid than the serum fraction. Similar studies were not carried out using monkey aorta. In the present study, we have observed that stearic and palmitic acid were more in intima than in media while linoleic and oleic acids were more in media than in intima in both human and monkey aorta. The high oleate/linoleate ratio is a typical feature of early atheromatous lesions (Swell *et al.*, 1960; Blomstrand and Christensen, 1961). It was observed in this study that this ratio was comparatively more in intima than in media. If we compare the fatty acid composition of different lipid components of monkey thoracic with abdominal aorta, then it was seen that the abdominal segment had more saturated and less unsaturated fatty acids as compared to thoracic aorta. This might explain the greater extent of atherosclerotic lesion in abdominal aorta as compared to thoracic aorta in this species.

As regards human aortic fatty acid composition, a similar trend was observed as in the case of monkey. If we compare fatty acid constituents of human thoracic aorta with monkey thoracic aorta, we find that saturated fatty acids are more in human aorta than in that of monkey, while a reverse relationship holds good with respect to unsaturated

fatty acids. Similar findings were noted when human abdominal aorta was compared with monkey abdominal aorta. The most striking difference observed in these two species was in the content of arachidonic acid. The phospholipid arachidonic acid was significantly higher in monkey thoracic and abdominal aorta as compared with corresponding regions of human aorta. The above findings may have important relevance to atherosclerosis since prostacyclin ( $\text{PGI}_2$ ) is the major product of arachidonic acid in walls of arteries and veins in several species including man (Nelson *et al.*, 1961). The most important properties of  $\text{PGI}_2$  so far elucidated are its vasodilator (Moncada *et al.*, 1976) and inhibitor action on platelet aggregation (Moncada and Vane, 1978).  $\text{PGI}_2$  applied locally in low concentration inhibits thrombus formation *in vivo* which is caused by ADP in the micro-circulation of hamsters cheek pouch (Higgs *et al.*, 1977). The greater quantity of arachidonic acid in monkey aorta may explain why this species develops less atherosclerosis and thrombosis as compared to that of human. One of the reports from this laboratory has already shown that incidence of naturally occurring atherosclerosis in monkeys is much less (27%), (Chakravarti and Kukreja, 1981) as against 80–90% in human aorta. Therefore, the present study has provided some evidence, on the basis of lipid constituents and fatty acid composition of the aorta from man and monkey, why incidences of spontaneously occurring atherosclerosis is much more in man than in the monkey.

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## Aortal collagen polymorphism in monkey and man

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**Abstract.** Aortal collagen typing in monkey and man showed the presence of types I, III and V in human aorta and types I and III in monkey aorta. Type III collagen was found to be a predominate type in both species. The molecular weight of type III collagen was similar in these species while type I collagen was different. Both monkey and human collagen types I and III were found to be immunogenic. Type I collagen was significantly increased while type III was decreased in human atherosclerotic plaque. Collagen typing in fatty streak remained unaltered.

**Keywords.** Human and monkey aorta; collagen types.

### Introduction

Collagen has been reported to be the major component of human atherosclerotic plaque and may account for as much as 60% of the intimal protein (Smith, 1965). This collagen is synthesised at an abnormally high rate by plaque cells (McCullagh and Ehrhart, 1974) and accumulates within the intima as the disease progresses. Since subhuman primates are similar to man phylogenetically and metabolically (Ganguly *et al.*, 1977; Chakravarti and Kukreja, 1981), a model of advanced atherosclerosis in this species has been developed in our laboratory (Kukreja *et al.*, 1981; Chakravarti, 1982). There are no reports regarding the distribution of collagen types in the aorta of rhesus monkeys. The present study was conducted to elucidate the biochemical and immunological similarities in aortal collagen polymorphism in monkey and man.

### Materials and methods

#### Chemicals

All the reagents used are of analytical grade. Pepsin was obtained from Sigma Chemical Company, St. Louis, Missouri, USA and diethylaminoethyl (DEAE)-cellulose-52 from Whatman Limited, England.

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\*To whom reprint requests to be sent.

Abbreviations used: DEAE, Diethylaminoethyl; SDS, sodium dodecyl sulphate.

*Selection of normal aorta*

Aorta samples were collected from 18 apparently normal healthy male rhesus monkeys and from 11 young human subjects who had died in traffic accidents. The body weight of rhesus monkeys was between 4–5 kg with an approximate age of 4 years and the age range for humans was between 25–35 years. At autopsy the whole aorta was opened longitudinally and examined with hand lens for fatty streaks and plaques. Those aortae which did not reveal macroscopic or microscopic lesions were employed for this study. These were washed with double distilled water and fibro-fatty adventitia was stripped off and media-intima processed for collagen separation.

*Selection of atherosclerotic aorta*

Atherosclerotic aorta from 5 human subjects who had died of atherosclerosis, ischaemic heart disease, hypertension and diabetes were taken. The aortae were examined with hand lens and Sudan IV dye for fatty streak and plaques and processed for collagen typing separately.

*Extraction procedure*

Extraction of collagen was done by the method of Moezer and Robert (1970) as modified by Robert *et al.* (1971). Briefly the aorta was cut into small fragments, delipidated with acetone and butanol, and homogenized in calcium chloride, Tris-citrate buffer, pH 7.5, using an Ultra-Turra homogeniser. This process was repeated 5–6 times. The supernatant was collected, pooled and dialysed against distilled water. After 48–72 h, the precipitate in the dialysis bag represented the crude collagen.

**Purification of collagen on DEAE-cellulose-52 column chromatography**

Crude collagen was purified according to the method of Timpl *et al.* (1978), where DEAE-cellulose-52 column (2.5 × 20 cm) was equilibrated in 2 M urea, 0.05 M Tris-HCl, pH 8.6 with a pressure of 80 mm Hg and flow rate of 10 ml/h. Approximately 80 mg of protein was charged and elution was carried out with linear salt gradient from 0.03 M NaCl. Before undergoing chromatographic procedure, the hydroxyproline content was estimated by the method of Slagemann and Stalder (1967).

*Chemical typing of collagen*

Aortal collagen typing was done according to the method of McCullagh *et al.* (1980). Purity of each collagen was further confirmed by polyacrylamide gel electrophoresis and compared with standard pattern of collagen separation already described by Sage *et al.* (1979).



### Raising of antisera

Antisera against crude collagen from monkey and human aorta was raised in rabbits according to the method of Ebisu *et al.* (1978). Five hundred  $\mu\text{g}$  of collagen was injected subcutaneously with Freund's complete adjuvant into two rabbits. Four injections at four different sites were given fortnightly and bleeding was done after a week of the last injection. Antibodies were detected by immunodiffusion technique.

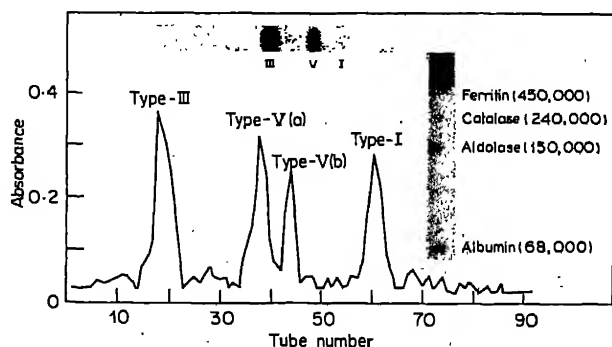
### Determination of molecular weights

Molecular weights of different types of collagen were determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Weber and Osborn, 1969) using ferritin, catalase, aldolase and albumin as marker proteins.

### Results

The crude collagen (Tris-citrate buffer extract) was fractionated on DEAE-cellulose-52 column chromatography and four major peaks were obtained, for both human (figure 1) and monkey (figure 2) aortal collagen, out of which peak I was common in both the species. Peak III of human aortal collagen corresponded with peak II of the monkeys. A comparison of polyacrylamide gel electrophoresis of different types of collagen and different peaks of column revealed that peaks I and IV of both the species corresponded to types III and I collagen respectively. Peaks II and III of human aortal collagen corresponded to types Va and Vb collagen respectively, whereas peaks II and III of monkey aorta collagen did not correspond to any of the five known collagen types (figures 1 and 2).

Table 1 shows collagen concentration ( $\mu\text{g}/\text{mg}$  dry weight) in human and monkey aorta. Type III collagen was found to be in maximum concentration in the aorta of both the species. There was complete absence of type V collagen in monkey aorta while it was present in human aorta at low concentration.



**Figure 1.** Human aortal collagen-DEAE-52 (2 M urea 0.05 Tris HCl buffer, pH 8.6; 0.03 M NaCl linear gradient, size  $20 \times 2.5$  cm, read at 230 nm).

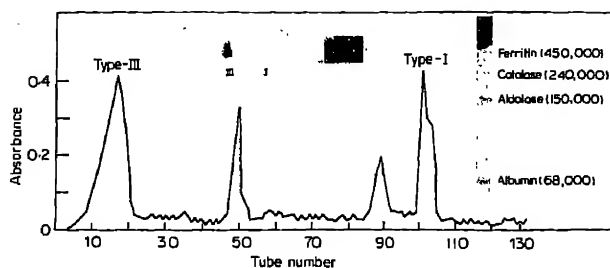


Figure 2. Monkey aortal collagen-DEAE-cellulose-52 (2 M urea 0.05 Tris HCl buffer, pH 8.6; 0.03 M NaCl linear gradient, size 20 × 2.5 cm, read at 230 nm).

Table 1. Collagen concentration ( $\mu\text{g}/\text{mg}$  dry wt).

Type	Human aorta	Monkey aorta
I	41.0 $\pm$ 7.2	37 $\pm$ 5.1
III	130 $\pm$ 10.7*	181 $\pm$ 9.3*
V	29 $\pm$ 3.5	—

\*  $P < 0.001$

Collagen concentration ( $\mu\text{g}/\text{mg}$  dry wt) in fatty streak and atherosclerotic plaque of human aorta is given in table 2. There was significant increase in type I collagen and decrease in type III collagen in fibrous plaque as compared to normal aorta. Concentration of type V collagen was slightly elevated in fibrous plaque. Collagen typing in fatty streak remained unaltered.

Molecular weight of different types of collagen is shown in table 3. Type I collagen was of a higher molecular weight than type III in the aorta of both species. Interestingly molecular weight of type III collagen from both species was found to be approximately similar.

Table 2. Collagen concentration in atherosclerotic human aorta.

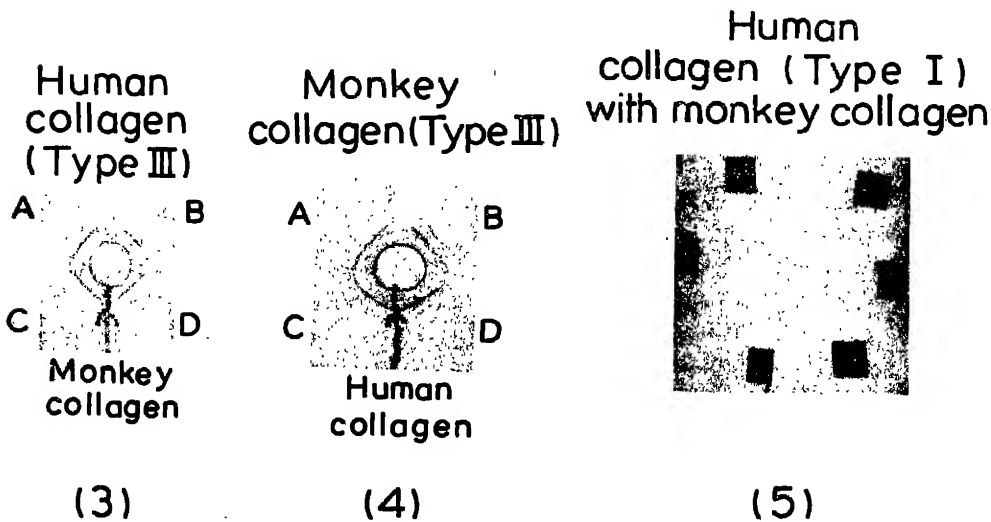
Tissue	Collagen concentration ( $\mu\text{g}/\text{mg}$ dry wt)		
	Type I	Type III	Type V
Normal human aorta	41.0 $\pm$ 7.2	130 $\pm$ 10.7	29 $\pm$ 3.5
Fatty streak	47.3 $\pm$ 8.7	129.8 $\pm$ 11.8	30.7 $\pm$ 4.1
Fibrous plaque	91.7 $\pm$ 10.8*	96.8 $\pm$ 6.5*	36.2 $\pm$ 4.9

\*  $P < 0.05$

**Table 3.** Molecular weights of different types of collagen through SDS polyacrylamide gel electrophoresis.

Type	Human aorta	Monkey aorta
I	210,000	140,000
III	107,000	105,000
V	145,000	—

Antisera raised in rabbits against crude collagen from human and monkey aorta cross-reacted with different types of collagen isolated from aorta of these species. Types I and III collagen reacted with crude collagen antisera of their respective species. Type V collagen of human aorta was found to be non-immunogenic. Further, the cross reactivity of different collagen types from the aortal of monkey and man showed that type III collagen of human aorta produced precipitin line against anti-monkey aortal collagen antibodies and vice versa and showed a line of identity with each other (figures 3 and 4) while type I failed to cross-react (figure 5).



**Figure 3-5.** 3. Cross-reactivity between human and monkey aortal collagen type III. Antiserum against monkey aortal collagen was put into the central well and type III collagen from human aorta, skin, cartilage and tendon were put into the wells, A, B, C and D respectively. 4. Cross-reactivity of type III collagen from human aorta with that of monkey. Antiserum against human aortal collagen was put into the central well and type III collagen from monkey aorta, skin, cartilage and tendon were put into the wells, A, B, C and D respectively. 5. Type I collagen from human aorta failed to cross-react with antiserum of crude monkey aortal collagen. Central well contains antiserum against monkey aortal collagen and wells, A, B, C, D and E contain type I collagen from human aorta, skin, cartilage, tendon and monkey aorta respectively.

## Discussion

Collagen is the major macromolecule of connective tissues and forms the structural component of both vascular and matrix tissue. Its synthesis is enhanced in atherosclerotic process by plaque cells (McCullagh and Ehrhart, 1974). Limited pepsin digestion extracted approximately 75% of the total aorta collagen and 80–90% of this extract was fractionated by salt precipitation technique (McCullagh et al., 1980).

In the present study, we have observed that type III collagen concentration is maximum in human and monkey aorta. In human atherosclerotic fibrous plaque, type I collagen concentration is significantly increased while type III collagen is decreased significantly, which suggest that a major shift in the nature of aortal collagen synthesis occurs in advanced atherosclerotic plaques (McCullagh and Balian, 1975). Miller (1978) reported trace amount of type IV collagen in aortic tissue, Barnes et al. (1978) contradicted it. We could not obtain type IV collagen in the normal aorta or fibrous plaque of either of these species. Recent literature has revealed the presence of another type of collagen from basement membrane in the aorta which has been termed as type V (McCullagh et al., 1980). We have also observed approximately 14% of type V collagen in normal human aorta and 16% in fibrous plaque respectively, but it was not detectable in the monkey aorta by the present method. However, chromatographic analysis revealed two protein peaks of monkey aortal collagen which were untypable by the salt fractionation method. There is no report in literature regarding collagen typing in monkey aorta. We have demonstrated the presence of types I and III collagen in the aorta of rhesus monkey using three different techniques viz. DEAE-cellulose column chromatography, polyacrylamide gel electrophoresis and limited pepsin digestion method. Absence of type V collagen in monkey aorta is an interesting finding.

To check for the similarity and dissimilarity of different types of collagen from human and monkey aorta, their molecular weights, immunogenicity and cross reactivity were assessed. It was found that the molecular weight of type III collagen in both species was similar but molecular weight of type I collagen was different. Types I and III collagens from monkey and human aorta were found to be immunogenic. Type V collagen of human aorta could not produce antibodies which may be due to its relatively low concentration in the aorta. So far as immunologic property is concerned, type III collagen of human and monkey aorta were antigenically similar, but type I collagen was quite different.

It appears that there are many similarities in the chromatographic pattern, molecular weight, antigenicity and chemical nature of human and monkey aortal collagens.

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## Purification and characterization of Cu-Zn superoxide dismutases from mungbean (*Vigna radiata*) seedlings

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**Abstract.** Mungbean contains three isoenzymes of superoxide dismutase designated isoenzyme I, II and III. The two cytosolic superoxide dismutases (I and II) were purified to homogeneity by ammonium sulphate fractionation, ion exchange chromatography on diethylaminoethyl cellulose, gel filtration and preparative polyacrylamide-gel electrophoresis. The molecular weights of isoenzyme I and isoenzyme II were determined to be 33,000 and 31,600 respectively. The subunit molecular weight was approximately 16,000 indicating that the isoenzymes contained two identical subunits. The ultra-violet absorption spectra revealed a maximum at 258–264 nm for the two isoenzymes. Superoxide dismutase I and II were inhibited to different extents by metal chelators. Isoenzyme I was more sensitive to inhibition by cyanide and azide, while isoenzyme II was more susceptible to inhibition by diethyldithiocarbamate and *o*-phenanthroline. Both the isoenzymes exhibited similar denaturation profiles with heat, guanidinium chloride and urea. The denaturation with urea and guanidinium chloride was reversible. The two copper-zinc enzymes were more stable towards thermal inactivation compared to manganese and iron superoxide dismutases from other sources. The results indicate that the two isoenzymes differ from each other only with respect to charge and sensitivity towards metal chelators.

**Keywords.** Superoxide dismutases; mungbean; isoenzymes.

### Introduction

Superoxide dismutase (SOD, EC 1.15.1.1) is an essential enzyme for the survival of oxygen utilizing organisms. It protects the cells against the toxic effects of superoxide radicals (Fridovich, 1978). Based on the metal content, three distinct classes (copper-zinc, manganese and iron) of this enzyme have been described (Fridovich, 1975). All the three enzymes were purified and characterized from diverse plant and animal sources (McCord, 1979; Salin and Bridges, 1980; Baum and Scandalios, 1981).

Among germinating seeds, of the three isoenzymes present in wheatgerm two copper-zinc enzymes were purified (Beauchamp and Fridovich, 1973), while only one Cu-Zn SOD was purified from greenpea (Sawada *et al.*, 1972). During the early stages of germination of mungbean seeds, we observed three distinct SOD of which SOD-I and SOD-II were copper-zinc enzymes while SOD-III is a manganese containing enzyme localized in the mitochondria (Reddy and Venkaiah, 1982). The present work describes the purification of the two Cu-Zn SOD and their characterization.

Abbreviations used: SOD, Superoxide dismutases; DDC, diethyldithiocarbamate; GdmCl, guanidinium chloride; UV, ultra-violet; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; *M<sub>r</sub>*, Molecular weight; DEAE, diethylaminoethyl.

## Materials and methods

SOD was assayed by the method of Mishra and Fridovich (1972) at pH 9.8 using Shimadzu-ultra-violet (UV) 180 double-beam spectrophotometer. Protein was estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard. In the last step of purification the protein concentration was determined according to the method of Murphy and Kies (1960). Disc gel electrophoresis of SOD isoenzymes was performed under non-denaturing conditions as described by Davis (1964). The enzyme activity was localized in the gels according to the photochemical method of Weisiger and Fridovich (1973). Slab gel electrophoresis was carried out on sodium dodecyl sulphate (SDS) permeated polyacrylamide gels (12.5%) as described by Laemmli (1970) to determine the molecular weight ( $M_r$ ) of the subunits. Phosphorylase b, BSA, ovalbumin, trypsinogen and lysozyme were used as  $M_r$  markers. The native  $M_r$  of the two SOD was determined by gel filtration on a calibrated Sephadex G-100 column (1.5 × 95 cm). Copper and zinc content of the two isoenzymes was determined by using a Varian-Techtron-Model 1000 atomic absorption spectrophotometer and the metal content was calculated as described by Sawada *et al.* (1972). All the purification steps were carried out at 0–4°C.

## Results

### *Purification of SOD-I and SOD-II*

**Crude extract:** Viable mungbean seeds (300 g) were surface sterilized with 0.1% mercuric chloride. Excess mercuric chloride was washed off with water and the seeds were allowed to germinate in the dark at 30°C ± 2 in plastic trays for 48 h. The seedlings (about 950 g) were washed with distilled water, chilled and homogenized in five batches with about 250 ml each of 0.01 M sodium phosphate buffer, pH 7.6. The homogenate was passed through two layers of muslin cloth and centrifuged for 30 min at 4,000 *g* in a Sorvall RC-5B centrifuge. The resulting supernatant was designated as the crude extract. To the crude extract 1 M manganous sulphate was added with constant stirring to give a final concentration of 10 mM. After 20 min the precipitated nucleoproteins were removed by centrifugation at 4,000 *g* for 30 min.

**Ammonium sulphate fractionation:** To the supernatant, solid ammonium sulphate was added to 35% saturation with constant stirring over a period of 20 min. After standing at 0–4°C for 15 min the solution was centrifuged for 45 min at 4,000 *g*. The precipitate was discarded and the SOD activity was precipitated from the supernatant by a further addition of solid  $(\text{NH}_4)_2\text{SO}_4$  to 60% saturation. After 15 min the solution was centrifuged (4,000 *g* for 45 min) and the pellet was dissolved in 0.01 M sodium phosphate buffer pH 7.6 and dialysed against the same buffer for 48 h.

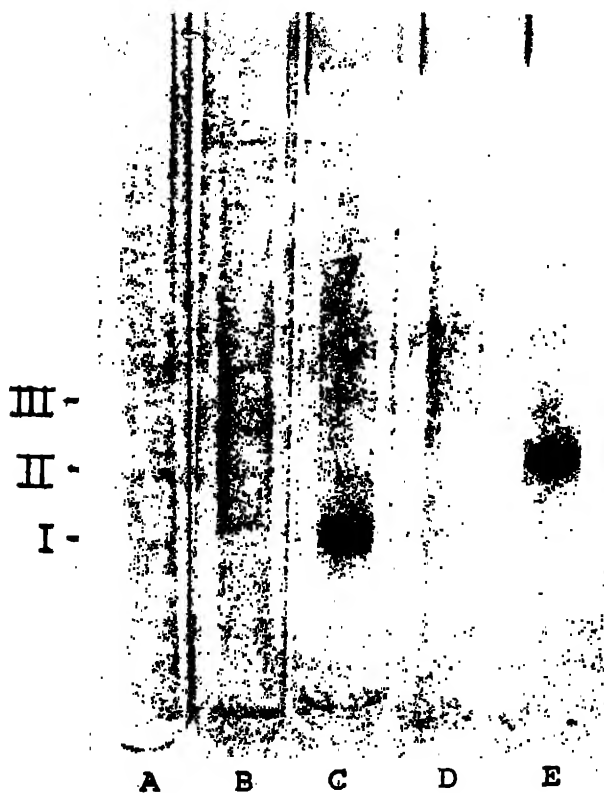
**Ion exchange chromatography:** The dialysed enzyme was applied on to a column (4 × 27 cm) of diethylaminoethyl (DEAE) cellulose pre-equilibrated with 0.01 M sodium phosphate buffer, pH 7.6. The SOD activity was eluted with the same buffer using 0–0.5 M NaCl gradient at a flow rate of 35 ml/h. Fractions containing SOD



activity were pooled and dialysed against 0.01 M phosphate buffer and adsorbed on to a column (2.3 × 23 cm) of DEAE-cellulose and eluted with 0.025 M NaCl gradient in a total volume of 800 ml. Every 4th fraction was screened for SOD activity by disc gel electrophoresis and activity staining (Weisiger and Fridovich, 1973).

**Gel filtration on Sephadex G-75:** Fractions containing isoenzyme I or isoenzyme II were pooled separately, concentrated against solid sucrose, applied on to a column (1.5 × 120 cm) of Sephadex G-75 pre-equilibrated with 0.01 M sodium phosphate buffer, pH 7.6 containing 0.02 M potassium chloride and eluted with the same buffer. The fractions containing either SOD-I activity or SOD-II activity were pooled and subjected to preparative electrophoresis.

**Preparative electrophoresis:** Isoenzyme I and II obtained from the gel filtration step were about 80% pure. Minor contaminants were removed by preparative disc gel electrophoresis (0–4°C) on 7.5% gels. A guide strip of the gel was stained with Coomassie blue-G for 20 min and matched with the unstained gels. The regions



**Figure 1.** Polyacrylamide gel electrophoresis of mungbean SOD. The gels were stained for activity (Weisiger and Fridovich, 1973) and protein. A. Crude extract containing 250  $\mu$ g of protein stained for SOD activity. B. Activity staining with 4  $\mu$ g of SOD-I. C. Protein staining with 140  $\mu$ g of SOD-I. D. Activity staining with 4  $\mu$ g of SOD-II. E. Protein staining with 140  $\mu$ g of SOD-II.

Table 1. Purification of Cu-Zn SOD from mungbean seedlings.

Purification Step	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude extract	50224	4.3	100	1
35-60% Ammonium sulphate precipitate	32100	15	64	4
DEAE-Cellulose-1	19872	48	40	11
DEAE-Cellulose-2				
Isoenzyme-I	7316	124	15	29
Isoenzyme-II	9516	156	19	36
Sephadex-G-75 Isoenzyme-I	4248	1180	9	274
Isoenzyme-II	6426	1530	13	356
Preparative electrophoresis-I	3936	1640	8	381
-II	5907	1846	12	429

One unit of SOD activity is defined as the quantity of enzyme that inhibits adrenaline autoxidation by 50% under the standard assay conditions (Mishra and Fridovich, 1972).

corresponding to the stained protein bands were cut and the enzyme from the gel was eluted by homogenising with 0.01 M phosphate buffer pH 7.6. The gel material was removed by centrifugation and the clarified supernatant was dialysed against 0.01 M phosphate buffer pH 7.6 for 24 h. The homogeneity of the two superoxide dismutases were tested by electrophoresis on 7.5% polyacrylamide disc gels. A single stainable protein band was observed (figure 1) and in each case it corresponded to the achromatic zones observed during activity staining. The total yield of SOD-I and SOD-II by the above procedure was 8% and 12% respectively with a fold purification of about 400 (table 1).

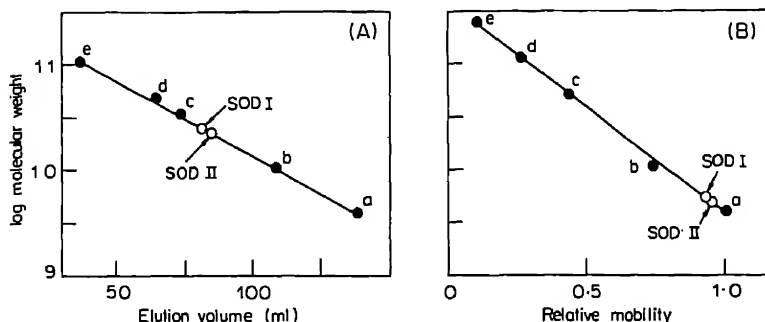
### Characterization

*M<sub>r</sub> and subunit composition:* The *M<sub>r</sub>* of the purified SOD-I and SOD-II were determined to be 33,000 and 31,600 respectively using a calibrated column of Sephadex G-100 (figure 2A). On SDS polyacrylamide gels (12.5%) in presence of 2.5% 2-mercapto-ethanol SOD-I and SOD-II exhibited *M<sub>r</sub>* of 16,500 and 16,000 respectively (figure 2B) indicating that the native isoenzymes were dimers.

*UV absorption spectrum:* UV absorption spectra of isoenzyme I and II were recorded at a concentration of 160 µg/ml and 180 µg/ml respectively. Both the isoenzymes exhibited an absorption maxima at 258-264 nm region indicating that phenylalanine was the predominant aromatic amino acid.

### Effect of inhibitors

Cyanide inhibited isoenzyme I and isoenzyme II at very low concentrations. The



**Figure 2.** A. The  $M_r$  determination of SOD-I and SOD-II by gel filtration on Sephadex G-100. The marker proteins 1 mg each were dissolved in 1.0 ml of 0.01 M sodium phosphate buffer pH 7.6 containing 0.02 M KCl along with SOD-I and SOD-II and applied on to the column. The fractions were monitored by measuring the absorbance at 280 nm for marker proteins and by activity measurements for SOD-I and SOD-II. In the order of increasing molecular weight the following marker proteins were used. (a) Lysozyme; (b) trypsinogen; (c) lactalbumin; (d) ovalbumin and (e) bovine serum albumin.

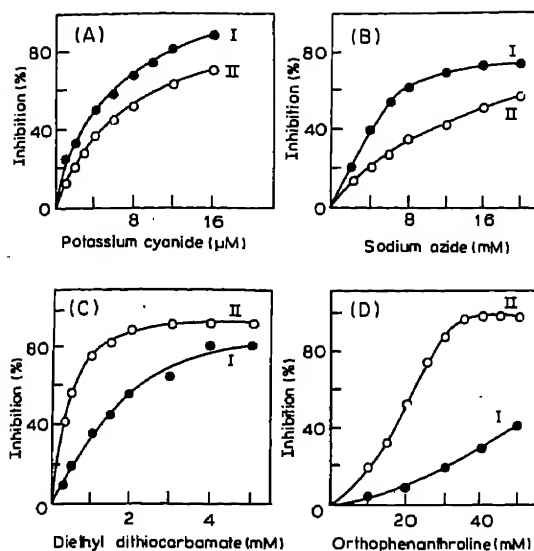
B. Subunit  $M_r$  determination on SDS polyacrylamide (12.5%) slab gel. Fifteen  $\mu$ g of marker proteins and SOD-I and SOD-II were heated for 5 min in 1% SDS containing 2.5% 2-mercaptoethanol and applied on to the gel. After the electrophoresis the gel was stained with Coomassie blue-R. In the order of increasing molecular weight the following marker proteins were used. (a) lysozyme; (b) trypsinogen; (c) ovalbumin; (d) bovine serum albumin; and (e) phosphorylase b.

inhibition of SOD activity observed with cyanide was rapid and did not require preincubation. The final concentration of cyanide in the assay mixture varied from 1  $\mu$ M to 16  $\mu$ M. Cyanide concentration required for 50% inhibition of SOD activity was found to be 4.5 and 7.8  $\mu$ M (extrapolated from the plot) for SOD-I and SOD-II respectively (figure 3A).

Azide did not interfere with adrenaline autoxidation employed for assaying SOD activity. Unlike cyanide, the inhibitory effect of azide was observed only when the enzymes were preincubated for 15 min. Figure 3B shows the results obtained when the enzymes were incubated at 30°C with varying concentrations of azide. From the inhibition pattern the concentration of azide required for 50% inhibition of SOD-I and SOD-II was calculated to be 5.5 and 16 mM respectively.

Since cyanide and azide inhibited the isoenzymes, the effect of diethyldithiocarbamate (DDC) which chelates specifically copper was tested. The inhibition was determined by incubating the isoenzymes with varying (0–5 mM) concentrations of DDC for 15 min in 20 mM bicarbonate buffer pH 9.8. Thirty  $\mu$ l of this incubation mixture was diluted to 3 ml of assay buffer (0.05 M bicarbonate buffer pH 9.8 and 0.1 mM EDTA) to avoid interference of the inhibitor with adrenaline autoxidation. SOD-II was more sensitive to inhibition by DDC than SOD-I (figure 3C). The concentration of DDC required for 50% inactivation of SOD-I and SOD-II was found to be 1.7 and 0.4 mM respectively.

The sensitivity of the two superoxide dismutases was compared by using *o*-phenanthroline as an inhibitor. SOD-I and SOD-II were incubated at 30°C with different concentrations (0–50 mM) of *o*-phenanthroline in 20 mM bicarbonate buffer



**Figure 3. A.** Effect of potassium cyanide on the activity of mungbean SOD. The assay mixture contained 0.05 M bicarbonate buffer pH 9.8, 1 mM EDTA, 720 ng of SOD-I(●) or 770 ng of SOD-II(O) and 0–16  $\mu\text{M}$  potassium cyanide. The reaction was started by the addition of 0.3 mM adrenaline. The inhibition of adrenaline autooxidation by SOD was followed at 470 nm for 3 min. using Shimadzu UV 180 spectrophotometer. The activity in the absence of cyanide was taken as 100%.

**B.** Inhibition of mungbean superoxide dismutases by sodium azide. Sodium azide (0–20 mM) was added to assay mixture containing SOD-I or SOD-II and incubated at 30°C for 15 min and the activity monitored at 470 nm.

**C.** Effect of DDC on mungbean SOD. The isoenzymes were incubated with different concentrations of DDC in 20 mM sodium bicarbonate-carbonate buffer pH 9.8 for 15 min at 30°C. Thirty  $\mu\text{l}$  aliquots were diluted into the assay mixture (3 ml) to avoid interference with adrenaline autooxidation. The SOD activity was monitored as described in A.

**D.** Effect of *o*-phenanthroline on mungbean SOD. SOD-I and SOD-II were incubated at 30°C with indicated concentrations of *o*-phenanthroline in 20 mM bicarbonate buffer pH 9.8 for 15 min and assayed for residual SOD activity after suitable dilution.

pH 9.8 for 15 min. An aliquot (30  $\mu\text{l}$ ) of the incubation mixture was diluted into 3 ml of assay mixture and assayed for SOD activity. *o*-Phenanthroline was a more potent inhibitor of SOD-II than SOD-I (figure 3D). Thus 30 mM *o*-phenanthroline inhibited SOD-I and SOD-II by about 20% and 90% respectively. Thus with DDC and *o*-phenanthroline the time dependent loss in SOD activity may be called inactivation rather than inhibition because both the isoenzymes were irreversibly inhibited even when the incubation mixture was diluted 100 times.

### Metal analysis

SOD-I and SOD-II were analysed for copper, zinc, manganese and iron using a Varian-Techtron-model 1000 atomic absorption spectrophotometer. The samples contained negligible amounts of manganese and iron. The results of metal analysis are given in

**Table 2.** Copper and zinc content of mungbean SOD analyzed by atomic absorption spectrophotometry. Comparison with wheatgerm and bovine erythrocyte SOD.

Metal content	Mungbean <sup>a</sup>		Wheatgerm <sup>b</sup>		Bovine erythrocyte <sup>c</sup> SOD
	SOD-I	SOD-II	SOD-I	SOD-II	
Atoms Cu/mol	1.44	1.60	1.5	1.6	1.94
Atoms Zn/mol	2.20	2.30	1.9	2.0	1.71

<sup>a</sup> Mungbean SOD-I corresponds to wheatgerm SOD-II with regard to mobility in polyacrylamide gels

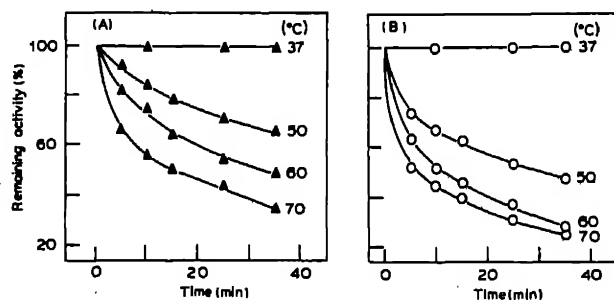
<sup>b</sup> Beauchamp and Fridovich (1973)

<sup>c</sup> McCord and Fridovich (1969)

table 2. The values are comparable with the copper and zinc content of superoxide dismutases from other sources (Beauchamp and Fridovich, 1973; McCord and Fridovich, 1969). Both the enzymes probably contain 2 atoms of zinc and 2 atoms of copper per molecule.

### Thermal inactivation

SOD-I and SOD-II (50 units) were incubated at different temperatures in 20 mM sodium bicarbonate-carbonate buffer pH 9.8. An aliquot required for 3 assays was removed at different time intervals and kept in ice for the estimation of residual activity. The results are shown in figure 4. The inactivation follows a first order reaction rate. A straight line was obtained when the log residual activity was plotted against time. From the slope of such a plot (not shown) at 70°C  $t_{1/2}$  was calculated to be 27 and 21 min for SOD-I and SOD-II respectively.



**Figure 4.** Thermal inactivation of mungbean SOD. Isoenzyme-I (A) and isoenzyme-II (B) about 50 units each were incubated in 20 mM bicarbonate buffer pH 9.8 at different temperatures. An aliquot required for three assays was removed at intervals shown in figure and stored in ice. Residual activity was assayed along with a zero time control.

*Denaturation by guanidinium chloride (GdmCl) and urea*

GdmCl was an effective denaturant of mungbean SOD when present in the assay medium. Thus in 0.6 mM GdmCl SOD-I was 46% active while SOD-II was 37% active. This denaturation was reversible, because enzymes incubated in 6 M GdmCl for 3 h at 30°C retained full activity when diluted into the standard assay mixture but not containing GdmCl.

The two copper zinc superoxide dismutases were stable towards urea denaturation. The two isoenzymes followed a similar pattern of denaturation. In 6 M urea SOD-I retained 60% of its activity while SOD-II retained about 50% of its activity. This effect of urea was reversible.

**Discussion**

Superoxide radicals ( $O_2^-$ ) are extremely reactive and toxic to the cellular organelles. SOD protect the cells by converting  $O_2^-$  into a less toxic metabolite,  $H_2O_2$ . Copper-zinc SOD isolated from various sources have been well characterized and shown to have sequence homology in the case of human, bovine, equine and yeast (Barra *et al.*, 1980). In this report an attempt is made to compare the two copper-zinc SOD purified to homogeneity from mungbean seedlings. The two isoenzymes are similar to each other in many properties and resemble the Cu-Zn SOD isolated from higher plants (Beauchamp and Fridovich, 1973; Sawada *et al.*, 1972; Asada *et al.*, 1977) and animals (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973). The  $M_r$  33,000 and 31,600 observed for SOD-I and SOD-II respectively is within the range of  $M_r$  observed for many copper-zinc SOD. Both isoenzymes have two identical subunits and absorb maximally at 258–264 nm indicating that phenylalanine is the predominant amino acid. In this respect the mungbean Cu/Zn SOD are similar to wheatgerm, greenpea and maize superoxide dismutases (Beauchamp and Fridovich, 1973; Sawada *et al.*, 1972; Baum and Scandalios, 1981).

Cyanide an effective inhibitor of bovine erythrocyte (Rotilio *et al.*, 1972) and spinach (Asada *et al.*, 1974) SOD, also inhibits mungbean SOD-I and SOD-II although at much lower concentrations. The inhibition is instantaneous indicating that cyanide has high affinity for the copper in the enzyme (figure 3A). Similarly azide inhibits mungbean SOD-I and SOD-II. SOD-I is more susceptible to inhibition by cyanide and azide when compared to SOD-II. This may be due to the difference in the exposure of copper to the solvent or due to the microenvironment of copper at the active site.

DDC which is a specific copper chelator inactivated the mungbean SOD, but SOD-II was more susceptible (figure 3C). Similarly SOD-II is more sensitive to inhibition by *o*-phenanthroline than SOD-I (figure 3D). Such a difference in the susceptibility towards various metal chelators reflects a difference in the accessibility of copper in the two isoenzymes.

SOD-I and SOD-II are stable towards thermal inactivation like the bovine erythrocyte SOD (Forman and Fridovich, 1973) but the Fe or Mn SOD isolated from various other sources (Puget and Michelson, 1974; Lumsden *et al.*, 1976) are less stable when compared to the two mungbean isoenzymes. The denaturation by GdmCl and

urea was reversible for the mungbean isoenzymes. SOD-I was more resistant towards these denaturants than SOD-II. In contrast this denaturation was not reversible in the case of Fe and Mn SOD from *Photobacterium sepia* and *Photobacterium leiognathi* (Puget and Michelson., 1974; Lumsden *et al.*, 1976). These results indicate that Cu-Zn SOD are more stable than Fe or Mn SOD

Our results indicate that although the two mungbean Cu-Zn SOD are closely related to each other, there exists subtle differences in the properties between the two isoenzymes that may be related to the charge difference of the two proteins or the accessibility of copper present at the active site for interaction with substrate or inhibitors.

## Acknowledgements

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## An indomethacin sensitive suppressor factor released by macrophages of leprosy patients

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**Abstract.** Reduction in  $F_c$  receptor expression as assayed by 'erythrocyte' rosetting of macrophage cultures from long term treated lepromatous leprosy patients (bacteriologically negative) was seen in the presence of viable *Mycobacterium leprae*. Macrophages with and without intracellular bacilli demonstrated this reduction. On the basis of this observation the conditioned medium of *Mycobacterium leprae* infected macrophage cultures of lepromatous patients, were tested on macrophages from normal individuals for [ $^3$ H]-leucine incorporation and antigen specific physical interaction with lymphocytes. Both these parameters showed decreased values as compared to the controls which were not exposed to this conditioned medium. Lymphocyte transformation to *Mycobacterium leprae* in leucocyte cultures of normal individuals was also reduced in the presence of the conditioned medium from lepromatous patients' macrophages. The indication that this factor may be a prostaglandin was suggested by the observation that its synthesis was inhibited by indomethacin. Its importance in the non-specific depression in cell-mediated immunity seen in lepromatous patients is discussed.

**Keywords.** Macrophage; lepromatous leprosy; prostaglandins.

### Introduction

An aberrant macrophage response to *Mycobacterium leprae* in lepromatous leprosy patients was reported earlier by us. Amongst these were a reduced ability to express  $F_c$  receptors, a significant reduction in protein synthesis and a negative macrophage-lymphocyte interaction in the presence of *M. leprae*. It was observed specifically in the first two systems that macrophages containing intracellular *M. leprae* seemed to exhibit a suppressive effect on macrophages within the same culture not harbouring intracellular bacilli (Birdi *et al.*, 1979, 1983).

The experiments presented here provide indications for the presence of a suppressor factor released by infected lepromatous macrophages, whose activity is recoverable from the spent culture medium. Various workers have demonstrated the indomethacin sensitive nature of prostaglandin synthesis (Goodwin, 1981). On the basis of the inhibition of the factor by indomethacin it could be suggested that this factor was a prostaglandin. This factor is distinct from the intracellular factor not affected by indomethacin reported previously (Salgame *et al.*, 1983).

Abbreviations used: BI+ve, Bacteriologically positive; BI-ve bacteriologically negative; EA rosetting, erythrocyte rosetting; SRBC, sensitized sheep erythrocytes; MEM, minimal essential medium; SI, stimulation index.

## Materials and methods

### *Choice of patients*

Leprosy patients were classified according to the Ridley and Jopling classification (Ridley and Jopling, 1966). Lepromatous patients have been further subdivided as bacteriologically positive (BI +ve) *i.e.* those that harbour acid-fast bacilli in skin smears, and bacteriologically negative (BI -ve) *i.e.* those patients who do not show any acid-fast bacilli in skin smears.

### *Source of M. leprae*

Biopsies of nodules from lepromatous patients were homogenized and then trypsinized. The *M. leprae* thus obtained after differential centrifugation was washed with saline, stored at 4°C and used within a week (Ambrose *et al.*, 1978).

### *Macrophages*

Mononuclear cells were isolated from heparinized peripheral blood by sedimentation in 6% Dextran. Macrophages were freed from most of the other cells by adherence to glass. The macrophages thus obtained were maintained for 7 days in minimal essential medium (MEM) containing 40% human AB serum. The culture medium was changed every 48 h.

### *Preparation of conditioned medium*

Macrophage cultures maintained for 7 days from lepromatous patients were infected *in vitro* with  $5 \times 10^6$  *M. leprae*/tube as enumerated by the method of Hanks *et al.* (1964). After 24 h the excess *M. leprae* was washed off and the culture was maintained for an additional 48 h after which the medium was collected free from cells. The conditioned medium obtained from  $0.3 \times 10^6$  lepromatous macrophages was added to each normal culture.

### *F<sub>c</sub> mediated erythrocyte rosetting ('EA' rosetting)*

*M. leprae* ( $5 \times 10^6$ ) were added to each Leighton tube culture. The cultures were incubated for 24 h before the excess *M. leprae* was washed off. Macrophages were further maintained for 72 h after *M. leprae* infection before 'EA' rosetting was carried out, using sensitized sheep erythrocytes (SRBC) (Birdi *et al.*, 1983). In brief, SRBC in a 2% suspension in MEM were sensitized with an equal volume of goat anti-SRBC antibody. A suspension of 1% sensitized SRBC was overlaid onto the macrophage monolayer and allowed to rosette for 30 min at 37°C under 5% CO<sub>2</sub>. Nonrosetted SRBC were removed by washing, and the monolayers were fixed in 2.5% glutaraldehyde and stained with Ziehl-Neelsen acid-fast stain to identify *M. leprae*. The

percentage of cells with two or more SRBCs attached was determined. A total of 200 cells were counted. The conditioned medium was added 24 h prior to 'EA' rosetting where required. The percentage of cells rosetted with three or more SRBCs was determined.

#### *Antigen specific macrophage-lymphocyte physical interaction*

Mononuclear cells from peripheral blood were isolated on a Ficoll-Triosil gradient. The cells so obtained consisted of 80% to 90% lymphocytes and 10–20% macrophages. The cells were then resuspended in MEM containing 20% human AB serum in a concentration of  $4 \times 10^6$  cells/ml and distributed into Leighton tubes containing coverslips. The conditioned medium was added along with  $3 \times 10^6$  *M. leprae*/tube and the cells were incubated at 37°C for 18 h. The non-rosetted lymphocytes were washed off and the cells were fixed in 2.5% glutaraldehyde and stained with Ziehl-Neelsen acid-fast stain. The percentage of macrophages with 2 or more lymphocytes adhering to it was determined.

#### *Effect of macrophage conditioned medium on [ $^3\text{H}$ ]-leucine incorporation by macrophages*

Macrophage monolayer cultures maintained for 7 days were exposed to the conditioned medium from various sources. Control cultures were maintained to which no conditioned medium was added *in vitro*. The cells were then labelled for 3 h with 1- $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-leucine (sp. act. 7.7 Ci/mmol) and further processed for scintillation counting. Radioactivity was measured in the trichloroacetic acid insoluble portion. Incorporation of the precursor added in control cultures was taken as the index of baseline incorporation in the macrophages. Per cent difference between experimental and control cultures was calculated.

#### *Lymphocyte transformation*

Mononuclear cells were separated over a Ficoll Triosil gradient. A cell count was taken by diluting with Turks fluid and the suspension was also checked for viability using 0.3% trypan blue. The cell suspension was adjusted to  $1 \times 10^6$  cells/ml in culture medium (MEM + 20% AB serum). Aliquots of 0.1 ml were distributed in each well of a microtitre plate. The *M. leprae* ( $3 \times 10^6$ /ml) was distributed in 0.1 ml amounts into each well. The cultures were harvested on the sixth day. Each culture combination was set up in triplicate. Eighteen h prior to harvesting the cultures, 1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-thymidine (sp. act. 9.8 Ci/mmol) was added to each well. Cells were processed for scintillation counting to determine the incorporation of [ $^3\text{H}$ ]-thymidine and the stimulation index (SI) was defined as the ratio of radioactivity incorporated in the experimental over that of control.

#### *Treatment with indomethacin*

Indomethacin (1  $\mu\text{g/ml}$ ) was added to the cultures for a period of 24 h prior to the assay.

### Treatment with cycloheximide

The effect of cycloheximide (2 µg/ml Sigma no. C 6255) was tested using 'EA' rosetting assay on macrophages of lepromatous (BI-ve) patients. Two protocols were used. In one set of experiments, *M. leprae* and cycloheximide were added simultaneously and incubated overnight. These cultures were maintained in medium containing cycloheximide after the excess antigen was washed off for 48 h after which 'EA' rosetting was done.

In a second set of experiments 24 h after *M. leprae* infection of the macrophage cultures, cycloheximide was added 48 h before 'EA' rosetting was carried out.

## Results

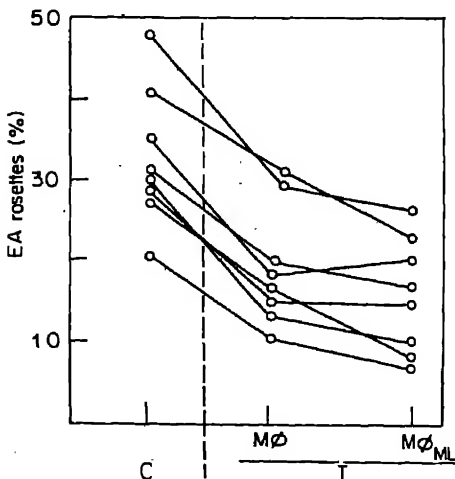
### Effect on 'EA'-rosetting

Macrophages from *M. leprae* infected cultures of lepromatous patients were divided into two populations—macrophages containing intracellular bacilli and those without. Since reduced rosetting was seen in both populations, it was probable that *M. leprae* interacted with the macrophages producing a factor that reduced the  $F_c$  activity of the macrophage in which it resided. Simultaneously these macrophages also secreted the inhibitory factor into the surrounding medium which reduced the rosetting capacity of other macrophages (figure 1).

This was confirmed by assessing the inhibitory function of the secreted factor on all the three macrophage parameters.

### Effect on [ $^3H$ ]-leucine incorporation into normal macrophages by the conditioned medium obtained from *M. leprae* infected macrophage cultures

The conditioned medium obtained from *M. leprae* infected macrophage cultures from



**Figure 1.** 'EA' rosetting of macrophages from lepromatous (BI-ve) patients in the presence of viable *M. leprae*. Each line represents 3 sets of values from a single patient. The variation in each vertical column represents the range of values. C, \*Control, uninfected macrophage culture. I, *M. leprae* infected macrophage culture. MØ, \*Macrophages with no intracellular *M. leprae*. MØ<sub>ML</sub>, \*Macrophage with intracellular *M. leprae*. a: c,  $P < 0.005$ ; b: c, not significant.

normal individuals and tuberculoid patients did not decrease the [ $^3\text{H}$ ]-leucine incorporation in normal macrophages (figure 2). However in the presence of the conditioned medium from *M. leprae* infected macrophage cultures of lepromatous patients, the [ $^3\text{H}$ ]-leucine incorporation of normal macrophages was reduced. If the macrophages from lepromatous (BI - ve) patients were infected with heat-killed *M. leprae*, no reduction in [ $^3\text{H}$ ]-leucine incorporation was noted.

*Effect on macrophage-lymphocyte normal interaction by the conditioned medium obtained from M. leprae infected macrophage cultures of lepromatous patients*

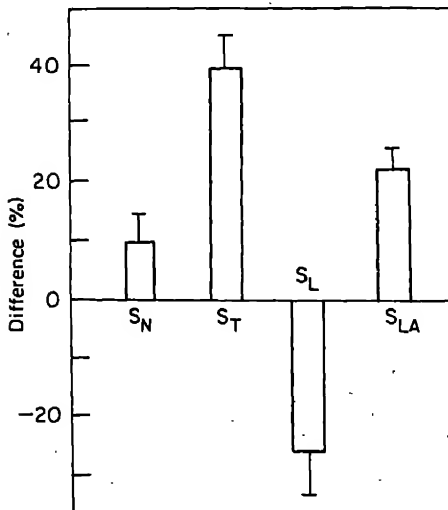
Normal interaction in the presence of antigen (*M. leprae*, PPD) was reduced if the lepromatous conditioned medium was introduced into the system but the levels were not lower than the baseline value of 10%. Therefore it appears that the factor in the conditioned medium is not specific in function (figure 3).

*Effect on lymphocyte proliferation*

*M. leprae* induced lymphocyte proliferation of normal individuals was inhibited in the presence of the conditioned medium prepared from lepromatous (BI - ve) macrophage infected *in vitro* with *M. leprae* (table 1). However the supernatant collected from lepromatous (BI - ve) macrophages alone had no suppressor activity.

*Effect of cycloheximide on the production of the inhibitory factor/s*

The requirement for protein synthesis by the macrophages from lepromatous (BI - ve) patients was assessed using a protein synthesis inhibitor-cycloheximide. It is evident from the data presented in figure 4A that the levels of 'EA' rosetting are restored in both



**Figure 2.** Effect of *M. leprae* infected macrophage supernatant on [ $^3\text{H}$ ]-leucine incorporation by normal macrophages. S<sub>N</sub>, Spent medium from macrophage cultures of normal subjects infected with viable *M. leprae*. S<sub>T</sub>, Spent medium from macrophage cultures of tuberculoid patients infected with *M. leprae*. S<sub>L</sub>, Spent medium from macrophage cultures of lepromatous patients infected with *M. leprae*. S<sub>LA</sub>, Spent medium macrophage cultures of lepromatous patients infected with autoclaved *M. leprae*. The results are expressed as mean  $\pm$  SE ( $n = 5$ ).

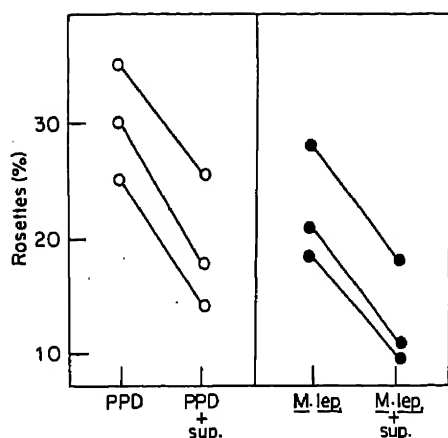


Figure 3. Effect of lepromatous macrophage supernatant on normal macrophage-lymphocyte interaction to *M. leprae* or purified tuberculin (PPD). Each line represents values from a single patient Sup, Conditioned medium from macrophage cultures of lepromatous patients infected with *M. leprae* PPD.

Table 1. The effect of the culture supernatant on *M. leprae* induced lymphocyte proliferation in normal individuals.

Type of supernatant added <sup>a</sup>	[ <sup>3</sup> H]-Thymidine incorporation <sup>b</sup> (mean cpm $\pm$ S.E.) <i>n</i> = 4	<i>P</i> value
Control	6432 $\pm$ 1277	
<i>M. leprae</i>	2503 $\pm$ 794	<i>P</i> < 0.05
Indomethacin	7404 $\pm$ 1583	NS
Indomethacin + <i>M. leprae</i>	7708 $\pm$ 1664	NS

<sup>a</sup>Supernatant was collected from bacillary negative lepromatous macrophages. Supernatant was assayed for suppressive activity in a normal lymphocyte proliferation assay to *M. leprae* antigen.

Control, Supernatant from macrophage cultures not exposed to *M. leprae*.

*M. leprae*, Supernatant from macrophage cultures exposed to *M. leprae*.

Indomethacin, Supernatant from macrophage cultures exposed to indomethacin only.

*M. leprae* + indomethacin, Supernatant from macrophage cultures exposed to *M. leprae* + indomethacin.

<sup>b</sup>The difference in response between normal peripheral blood mononuclear cells stimulated with *M. leprae* and the unstimulated background count.

macrophage populations *i.e.* those containing intracellular *M. leprae* and those without, if the drug is added along with *M. leprae*.

However if bacilli were added 48 h prior to the addition of cycloheximide, only the macrophages with no intracellular bacilli showed improved levels of rosetting (figure 4B). These kinetic results therefore implicate two factors that mediate suppression. The first is a stable early interaction product contained interacellularly. The second is continuously produced and secreted by the macrophages with intracellular *M. leprae* and mediates amplification.

#### Effect of indomethacin on the factor secreted by the macrophages

'EA' rosetting: The possibility that this factor was a prostaglandin was investigated by checking if its synthesis was blocked by indomethacin.

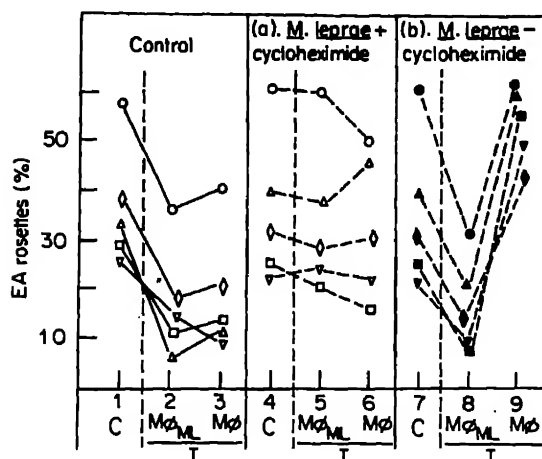


Figure 4. Effect of cycloheximide on the production of the inhibitory factor. *M. lep* + cycloheximide: Both added simultaneously. *M. lep* - cycloheximide: *M. leprae* added 24 h prior C, Uninfected macrophage cultures. I, *M. leprae* infected macrophage cultures.  $M\phi$ , Macrophages without intracellular *M. leprae*.  $M\phi_{ML}$ , Macrophages with intracellular *M. leprae*. (○), Untreated cultures; (●), culture treated with cycloheximide. Each symbol in all the 3 panels denotes a single patient. 1:4, Not significant; 5:6, not significant; 8:9,  $P < 0.005$ ; 4:5, not significant; 7:8,  $P < 0.005$ .

'EA' rosetting of macrophages from lepromatous (BI - ve) patients infected with *M. leprae* *in vitro* and treated with indomethacin was determined. With this reagent present in the culture, macrophages devoid of intracellular acid fast bacilli did not show any reduction in their capacity to form rosettes with sensitized SRBC. However, cells harbouring *M. leprae* continued to show the same low level of rosetting values similar to those seen in indomethacin free cultures (figure 5).

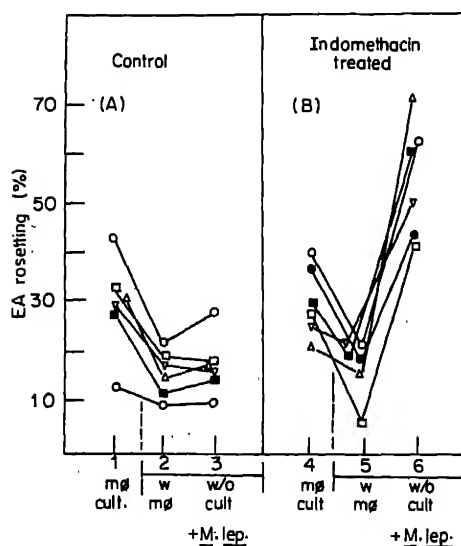


Figure 5. 'EA' rosetting of macrophages from lepromatous (BI - ve) patients. A. Control cultures. B. Indomethacin treated cultures. Each symbol represents one lepromatous patient. w, Macrophage with intracellular *M. leprae*. w/o, Macrophages without intracellular *M. leprae*.  $m\phi$  cult, Macrophage cultures from lepromatous (BI - ve) patients.  $m\phi$  cult. + *M. lep*, Macrophage cultures from lepromatous (BI - ve) patients infected with *M. leprae*. 1:2,  $P < 0.01$ ; 2:3, not significant; 5:6,  $P < 0.001$ ; 2:5, not significant; 3:6,  $P < 0.001$ .

**Lymphocyte proliferation assay:** The supernatants of indomethacin treated lepromatous cultures failed to show any inhibitory action on a normal *M. leprae* induced lymphocyte proliferation assay (table 1).

## Discussion

It is clear from the data presented that macrophages of lepromatous leprosy patients on incubation with *M. leprae* release a factor that alters some basic functions of normal macrophages.

The factor released in macrophage culture medium is specific for an interaction between viable *M. leprae* only and macrophages from a lepromatous patient. The depression was not seen with the conditioned medium from tuberculoid or normal individuals macrophages infected with  $5 \times 10^6$  *M. leprae*/culture or heat killed *M. leprae* and in earlier studies on  $F_c$  receptor expression (Birdi *et al.*, 1983).

Studies carried out in our laboratory have also demonstrated the presence of a specific intracellular suppressor factor in the lysed preparations of lepromatous macrophages infected with viable *M. leprae* (lysate) which was capable of altering normal macrophage function. The extracellular factor reported in this paper differs from the product retained within the infected macrophage (Salgame *et al.*, 1980).

Studies by Preston (1979) demonstrated 2 factors in *M. lepraemurium* infected mice, one able to activate and the other able to suppress. In resistant mice the activating factor played a major role while in susceptible mice the suppressor factor was dominant. It is possible that the increase in [ $^3\text{H}$ ]-leucine seen with the conditioned medium from tuberculoid patients is due to a similar factor.

Addition of indomethacin in our system did not increase 'EA' rosette values of cells containing bacilli, though it did augment 'EA' rosette formation in uninfected cells. In the former therefore there are two possibilities. The first is that the decrease in rosette forming cells in bacillary positive cells is not due to prostaglandin or that an intracellular pool of prostaglandin exists within the macrophage which reduces the rosetting capacity of the cell. If the latter were true, then the continuous release of prostaglandin formed prior to indomethacin addition would not be inhibited by an antagonist of prostaglandin synthesis and a depression would be apparent in the rosetting activity of the uninfected cells. This is not so (figure 5). Therefore this line of argument supports the first possibility that non-prostaglandin materials may be responsible only for reduced rosetting in the cells containing bacilli.

The kinetic results with cycloheximide show that the intracellular indomethacin resistant factor may be produced in the early stages of interaction since a lapse of 48 h between antigen addition and cycloheximide addition does not result in an increase of rosetting activity of cells harbouring acid fast bacilli. However cells not harbouring intracellular bacilli do not show any depression suggesting that the second factor is indomethacin sensitive and is continuously produced and secreted.

This subserves the notion that the two factors *viz.* lysate and prostaglandin act as independent entities and that suppression brought on by one is independent of the action of the other. Turcotte and Lemieux (1982) are supportive in their observations that in BCG infected mice the suppression is mediated by two different mechanisms.



The presence of a suppressor factor has also been reported by Satish *et al.* (1983).

Recent results obtained by Bahr *et al.* (1981) suggested that a normal prostaglandin dependent indomethacin sensitive regulatory mechanism was absent from the peripheral blood nonnuclear cells of lepromatous patients. Our data show that the production of a specific suppressor factor is not affected by indomethacin. It is therefore probable that the lymphocyte transformation test utilized by them detected in lepromatous (BI + ve) patients the indomethacin resistant factor as identified by us. It is conceivable that in these patients immunodepression would be modulated mainly through the specific suppressor factors that are not prostaglandins (lysate).

It is not clear whether the difference between the normal and lepromatous supernatant is qualitative or quantitative. It is probable that the amount of regulatory factor released by lepromatous macrophages far exceeds that produced by normal macrophages or that it makes an appearance at earlier stages of infection. Either way it culminates in a premature suppression of the immune response by macrophages unaffected by *M. leprae*, adding to the several abnormal sequence of events occurring inside the cells infected with *M. leprae*.

The possibility exists that the non-specific anergy seen in extremely bacilliferous lepromatous patients is due to the non-specific depression of the immune response by prostaglandins released by infected macrophages. On treatment, these lepromatous patients demonstrate a reduction in their bacillary load and regain their cell-mediated immunity to unrelated antigens but continue to show a specific anergy to *M. leprae*.

The importance of the regulation of the immune response by prostaglandins has been emphasized by the finding that the altered immune response in Hodgkin's disease may be caused by the increased production of prostaglandins (Goodwin *et al.*, 1977). Studies carried out by us have demonstrated an altered macrophage topography in lepromatous patients. These observations conform with those of Oropeza-Rendon *et al.* (1980) who have shown that prostaglandins alter the configuration of the macrophage membrane. Concanavalin A activated cells have shown that monocyte suppressive activity is predominantly mediated through the release of prostaglandin  $E_2$  which induces suppressor *T* cells. The induction of *T*-suppressor cells in lepromatous leprosy could be the result of a similar mechanism.

Studies carried out by Shand *et al.* (1981) demonstrate that *T* lymphocytes do not require to undergo proliferation or differentiation to function as suppressor cells since colchicine could prevent suppressor activity. This result indicates the probability that it is a membrane-linked phenomenon. Thus prostaglandin  $E_2$  maybe acting via this mechanism in the induction of suppressor *T* cells.

While prostaglandins appear to suppress cell mediated immunity directly, in humoral immunity they are necessary but not sufficient for inhibition since direct addition of prostaglandin to plaque forming cells *in vitro* is generally not suppressive but prostaglandin synthetase inhibitors enhance humoral responses (Robertson, 1981). It is accepted that in lepromatous leprosy the humoral response is not reduced and there is suppression only of CMI responses.

It is likely therefore that *M. leprae* not only evades the host immune defence system but also activates a suppressor mechanism *via* the host's own surveillance network. Prostaglandin synthesis may be one such mechanism that is affected.

## Acknowledgements

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## A versatile gel casting cum electrophoresis apparatus\*

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**Abstract.** A simple apparatus for vertical, *in situ*, polyacrylamide or agarose gel casting as well as for the subsequent electrophoresis is described. The apparatus is completely leakproof and does not require any special device like clamps, O-rings, gaskets, grease etc. for sealing. Slab gels of various thickness (0.04 to 1.0 cm) can be made and the apparatus can be used for analytical or preparative purposes. Gel rods can also be cast and run in the device. Forward as well as reverse polarity electrophoresis of a sample can be run simultaneously in the apparatus.

**Keywords.** Polyacrylamide gel electrophoresis apparatus; gel electrophoresis simple apparatus; gel casting cum electrophoresis; simultaneous forward and reverse electrophoresis.

### Introduction

Several models of vertical gel electrophoresis have been developed by different workers. Sophisticated commercial models are also available. In a recent comprehensive review on preparative gel electrophoresis, Chrambach and Nguyen (1979) have stressed the need for a simpler design for preparative gel electrophoresis which is free from the effects of mechanical and hydrostatic pressures on the gel. The equipment available including the commercial models possesses one or more of the following disadvantages: The gels are made separately in gel casting device and then transferred to the electrophoresis apparatus, thus developing mechanical stresses (Tichy, 1966; Akroyd, 1967; Studier, 1973; Bambeck and Black, 1981). Hydrostatic equilibrium is not attained (Tichy, 1966; Akroyd, 1967; Studier, 1973; Ogito and Market, 1979). Moreover to avoid leakage, the above mentioned models and also those described by Blatter (1969), Roberts and Jones (1972) and Andrew *et al.* (1979) require devices such as clamps, screws, O-rings, gaskets, melted agar, grease and plasticine clay.

This paper describes a simple and inexpensive, multipurpose apparatus for *in situ* gel making and subsequent electrophoresis which is free from the disadvantages mentioned above. An added advantage of this apparatus is that, with minor modifications, it

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Abbreviations used: PA, Polyacrylamide; PAGE, polyacrylamide gel electrophoresis; Bis, N,N'-methylenebis (acrylamide); TEMED, N,N,N',N'-tetramethylethylenediamine; UV, ultra-violet.

can also be used to obtain a total scan of biological extracts by running a simultaneous forward and reverse polarity electrophoresis.

## Materials and methods

### Chemicals

All common chemicals used were of the analytical reagent grade. The following chemicals and biochemicals were obtained from the sources indicated: myoglobin (from horse heart), trypsin (from bovine pancreas, Type III, EC 3.4.21.4), albumin (bovine serum, crystallized), amido black 10 B, ethidium bromide (crystalline) and ferritin (from horse spleen, Type 1) were from Sigma Chemical Co., St. Louis, Missouri, USA; and casein (Hammersten) was from E. Merck, Darmstadt, West Germany. The chemicals used for polyacrylamide gel electrophoresis (PAGE) such as acrylamide, N,N'-methylenebis acrylamide (Bis) and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Company, Rochester, USA. Glycine was from Kochlight Laboratories, Buckinghamshire, UK. Agarose (electrophoresis grade) was purchased from Sisco Research Laboratories, Bombay. DNA marker (Hind III digest of  $\lambda$  DNA) was a generous gift from Dr. S. Modak, Poona University, Poona. Culture filtrate from the fungus *Conidiobolus* (Srinivasan et al., 1983) was used for simultaneous forward and reverse electrophoresis. Perspex sheets were procured locally.

### Solutions for gel electrophoresis of proteins and nucleic acids

**Solutions for alkaline pH runs:** These were prepared according to Davis (1964). Both the upper and lower electrode buffers contained 0.005 M Tris and 0.04 M glycine, pH 8.3. The gel composition was 0.38 M Tris and 0.06 M HCl, pH 8.9; 7% acrylamide, 0.18% Bis, 0.03% TEMED and 0.07% ammonium persulphate.

**Solutions for electrophoresis at acidic pH values:** These were prepared according to Reisfeld et al. (1962) with slight modification. The two electrode buffers contained 0.04 M glycine and 0.0035 M acetic acid, pH 4.0. The gel composition was 0.36 M acetic acid and 0.06 M KOH, pH 4.3, 7% acrylamide, 0.1% Bis and 0.14% ammonium persulphate.

**Solutions for agarose gel electrophoresis:** These were prepared according to Thomas and Davis (1975). Both the electrode buffers contained 0.089 M Tris, 0.089 M boric acid and 2.5 mM EDTA, pH 8.2. Agarose gel composition was 0.8% agarose in the above buffer.

**Tracking dye:** Basic fuchsin was used for acidic runs and bromophenol blue for other runs.

**Composition of contact gel:** Contact gel was made in the bath buffer composition of the respective runs (e.g. contact gel composition for alkaline runs was: 0.005 M Tris and 0.04 M glycine, pH 8.3, 7% acrylamide, 0.18% Bis, 0.03% TEMED and 0.07%

ammonium persulphate. Contact gel composition for the simultaneous forward and reverse runs was: 0.005 M KCl, 7 % acrylamide, 0.18 % Bis, 0.06 % TEMED and 0.14 % ammonium persulphate. Contact gel can be reused several times by preserving the gel in cold under a layer of the buffer used for making it.

*Staining and destaining:* The gels were stained with amido black (0.5 % in 7 % acetic acid) and destained with 7 % acetic acid. Agarose gel was stained with ethidium bromide (1 mg/litre  $H_2O$ ) and the DNA markers were visualized over an ultra-violet (UV) transilluminator.

*Elution and assay of trypsin:* Trypsin was eluted from PAGE by the method of Bodhe *et al.* (1982) and estimated by the spectrophotometric assay of Kunitz using caseinolytic assay (Kunitz, 1947).

### Apparatus

*Design principle:* The apparatus is designed for vertical gel electrophoresis. It is made from perspex sheets. Figures 1 and 2 show the photographs of the empty and the assembled unit. In addition figures 3 and 4 give the dimensions of the unit.

The main apparatus consists of two vertical chambers which form the electrode compartments. These chambers are adjacent to each other and are separated by a common middle partition wall. A gap of 0.8 cm is kept at the base of the partition wall. One cm thick gel is cast at the base of the chambers which also closes the gap (0.8 cm) kept below the partition wall. Thus this basal 'contact gel' makes the two chambers leakproof (prevents the buffer flow from one chamber to the other), and also establishes an electrical contact between the two chambers.



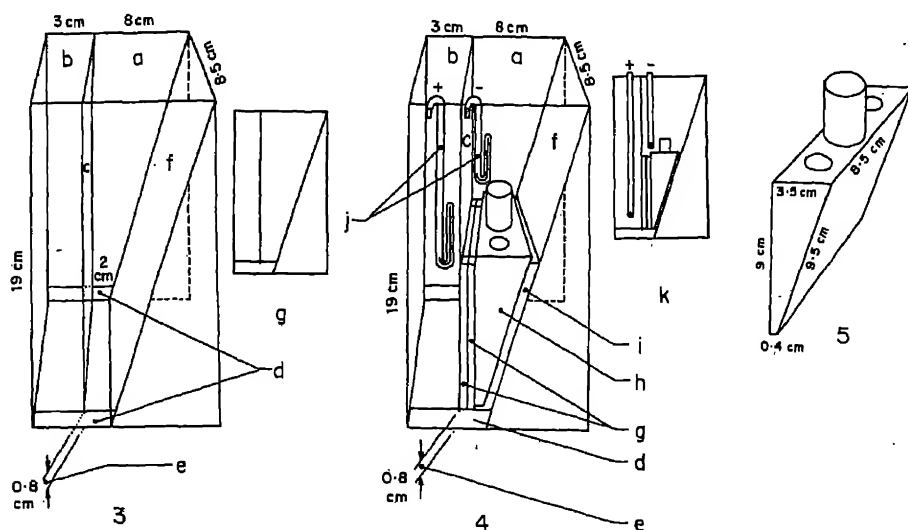
Figures 1 and 2. 1. Photograph of the empty unit. 2. Photograph of the assembled unit.

Gel cassettes (molds for casting slab gels) are placed above the contact gel in one of the chambers (chamber 'a') and are secured in position by a wedge (figures 4h and 5). Polyarylamide (PA) slab gels are cast in the gel cassettes, both the chambers are filled with buffer and after loading the sample, *in situ* electrophoresis is carried out since both the chambers also form the two electrode compartments. Thus out of the two chambers one chamber serves as a chamber for gel casting and both the chambers act as electrode compartments.

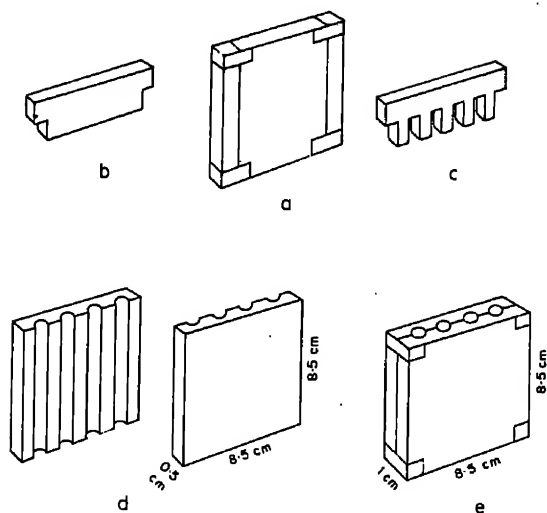
**Mode of construction:** In chamber 'a' where the gels are cast, the wall opposite the middle partition wall is made slanting for the operation of wedge. The middle partition wall is made of glass and sealed with araldyte against the walls of the equipment.

The wedge (figures 4h and 5) is made of perspex sheets, except for its surface facing the gel cassettes which is made of glass and sealed to the wedge with araldyte. The wedge is hollow and opens from the top only (see discussion).

Gel cassettes (glass, plates,  $8.5 \times 8.5$  cm and 0.1 cm thickness) and well former for slab gel are made as in the Pharmacia apparatus (GE-2/4). Figure 6 shows the details regarding the slab and rod gel cassettes. For making a slab gel cassette, two spacer strips



**Figures 3-5.** 3. Empty Unit. (a) Chamber with slanting wall, with  $2 \times 8.5$  cm base. (b) Second chamber,  $3 \times 8.5 \times 19$  cm; (c) Common middle wall,  $8.5 \times 18.2$  cm height; (d) Two support strips each  $5.5 \times 0.5 \times 1$  cm; (e) 0.8 cm gap below the common middle wall; (f) Slanting wall of chamber 'a'; (g) Elevation of the empty unit. [Thickness of the perspex sheet (0.5 cm) used in the construction of the main unit is not shown in the figure]. 4. Assembled Unit. (a) Chamber with slanting wall, with  $2 \times 8.5$  cm base; (b) Second chamber,  $3 \times 8.5 \times 19$  cm; (c) Common middle wall,  $8.5 \times 18.2$  cm height; (d) Two support strips, each  $5.5 \times 0.5 \times 1$  cm; (e) 0.8 cm gap below the common middle wall; (f) Slanting wall of chamber 'a'; (g) Gel cassettes; (h) Wedge; (i) Spacer or spacers if necessary, each  $8.5 \times 9.5$  cm height, thickness from 0.3 to 0.5 cm; (j) Two 'L' shaped platinum electrodes. The electrode in the sample chamber 'a' is held above and closer to the surface of the gel cassettes. Similarly the electrode in the other chamber is held closer to the contact gel; (k) Elevation of the assembled unit. 5. Wedge (with two circular openings and a handle on the top).



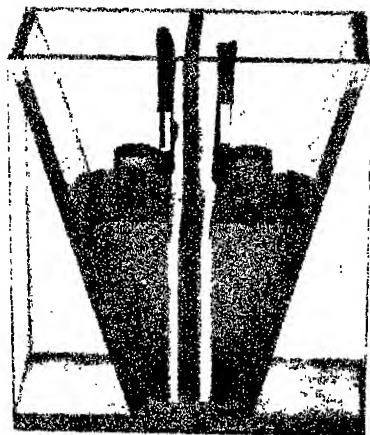
**Figure 6.** Gel cassettes. (a) Slab gel cassette; (b) Single well former for slab gel; (c) Multi well former for slab gel; (d) Two halves of the gel rod cassette (each  $8.5 \times 0.5 \times 8.5$  cm) exposing the half round grooves; (e) Gel rod cassette assembled,  $8.5 \times 1.0 \times 8.5$  cm.

are placed at the edges of a glass plate and the second glass plate is placed on them. The assembly is then held in position by two small strips of adhesive tape. The thickness of the gel can be varied as desired (0.04 to 1 cm) by using spacer strips of different thickness. For preparative type gel a 'single well former' is used. Spacers of different thickness and 'well formers' with different number of teeth can be easily cut from neoprene sheets.

Gel cassette for gel rod casting is made as shown in figure 6. It is made from two glass plates ( $8.5 \times 0.5 \times 8.5$  cm) in which four half round grooves of 0.3 cm radius are made on each plate. When both the plates are placed on one another four hollow tubes are formed inside. The rod 'well former' is made of cylindrical perspex teeth.

### Operating procedure

Forty ml of 7% 'contact gel' is polymerised at the base of the apparatus. This fills up the base and the basal gap between the two chambers and comes up to a 1 cm height and thus to the level of the support strips. One or more slab gel cassettes are placed in chamber 'a' at the base of the apparatus on the two support strips and above the contact gel. The cassettes are secured by the wedge (figure 4h). After polymerising the gel in the cassettes, both the chambers of the apparatus are filled with the bath buffer, sample is loaded, electrodes are positioned and the run is started by making electrical contacts. After completing the run the bath buffer in the chambers is poured out and after removing the wedge the gel slabs are taken out, stained, destained or if desired the proteins can be electrophoretically eluted (Bodhe *et al.*, 1982). Gel rod is removed simply by opening the two halves of the rod casting cassette.



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Figure 7. Photograph of the assembled unit for 'simultaneous forward and reverse electrophoresis'.

#### *Simultaneous forward and reverse polarity electrophoresis*

The above mentioned apparatus with a few modifications was used for simultaneous forward and reverse polarity electrophoresis. This design can be used for scanning biological extracts containing both acidic as well as basic proteins in a single run. The modification involves making chamber 'b' a replica of chamber 'a'. Consequently the unit will have two slanting walls and two wedges. Figure 7 shows the photograph of the assembled unit. In this apparatus the gels are cast in both the compartments (one gel in each compartment), and the same sample is loaded in both the compartments. Electrophoresis is carried out as mentioned in the earlier unit. Thus in this apparatus both the chambers serve as chambers for gel making as well as, as electrode compartments.

#### **Results**

Electrophoresis of marker protein, trypsin, DNA markers and culture filtrate of *Conidiobolus*.

#### *Electrophoresis under alkaline conditions*

**Normal size gels:** Two slab gels (each  $8.5 \times 0.3 \times 8.5$  cm) were run together. In one gel a mixture of 400  $\mu$ g each of ferritin, myoglobin, serum albumin and trypsin inhibitor were loaded. In the second gel the same marker mixture was loaded in three wells (15, 30



and 45  $\mu\text{g}$  of each protein respectively). The band pattern of the proteins stained is shown in figure 8.

*Thin gels:* Three gels ( $8.5 \times 0.04 \times 8.5$  cm) were run simultaneously in the apparatus. Fifteen per cent gels were made instead of 7%. In each gel, marker protein mixture (30  $\mu\text{g}$  of each marker) was loaded. After electrophoresis, gels were stained for 5 min in amido black and destained in 10 min by suspending in 7% acetic acid (figure 9).

*Thick gel:* Preparative run in 1 cm thick gel. One gel ( $8.5 \times 1.0 \times 8.5$  cm) was run. Marker mixture containing 7 mg of each marker protein (total 28 mg protein) was electrophoresed (figure 10).

*Gel rods of high PA concentration:* Four gel rods of 15% PA were cast in the cassette and a mixture of 20 to 40  $\mu\text{g}$  of each marker protein was loaded in each gel rod. After the run the rods could be easily taken out by simply opening the cassette (figure 11).

#### *Electrophoresis under acidic conditions*

*A labile protein:* Trypsin was run in a cold room at 5 to 8°C. Two slab gels ( $8.5 \times 0.3 \times 8.5$  cm) were run together. Two mg of trypsin was loaded in each gel. After the run, one gel slab was stained (figure 12) and the other gel was used for the electrophoretic elution of trypsin (Bodhe *et al.*, 1982), 78% of the trypsin activity was eluted.

#### *DNA markers in agarose gel*

One gel slab ( $8.5 \times 0.3 \times 8.5$  cm) containing 0.8% agarose gel with wells was run after loading 0.1  $\mu\text{g}$  of DNA marker (figure 13).

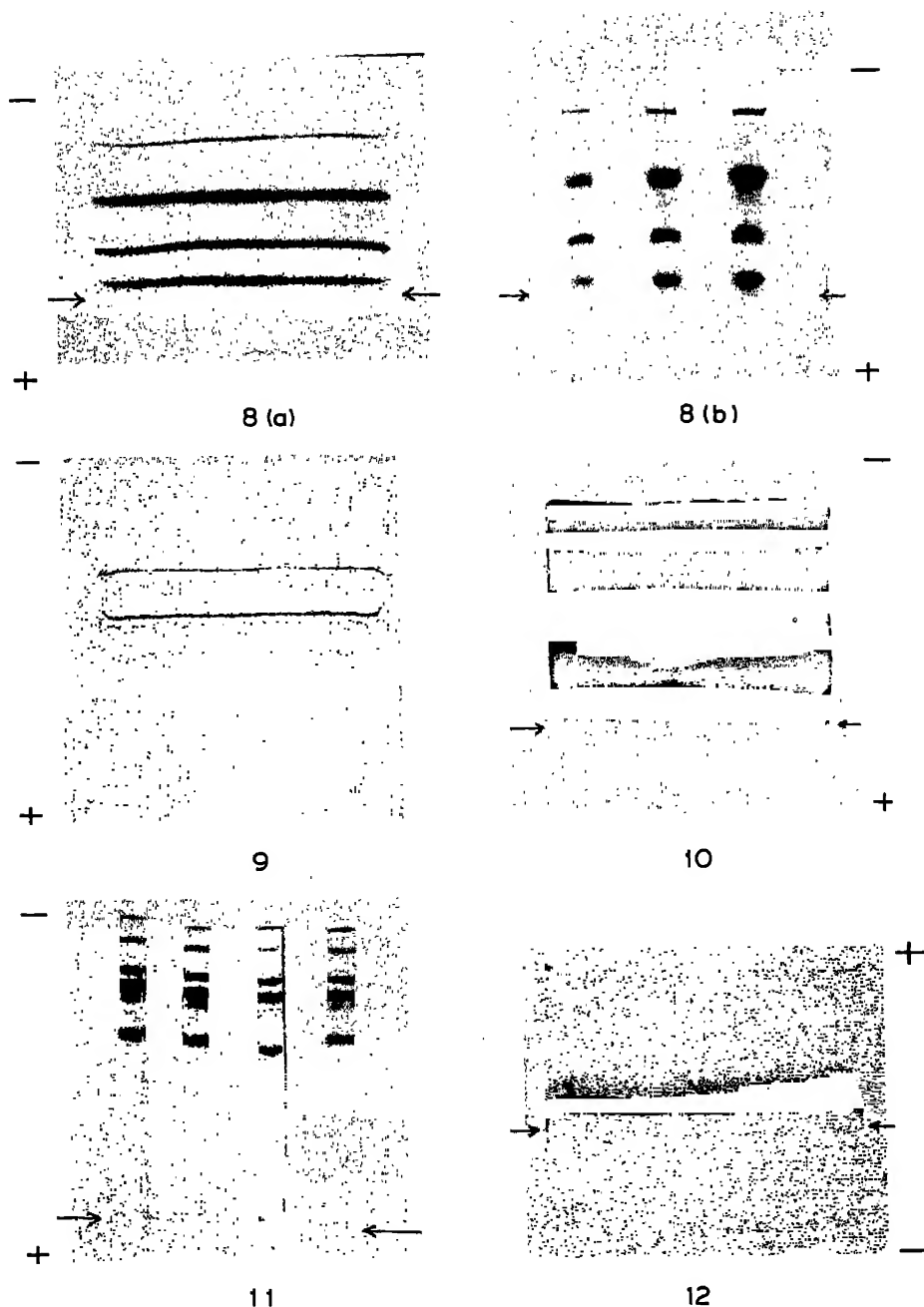
#### *Simultaneous forward and reverse polarity electrophoresis of fungal broth*

*Both forward and reverse runs at pH 8.9:* The gel and buffer compositions in both the chambers were those of pH 8.9 system. Broth sample (5 mg protein) was loaded in the gel of each chamber. Figure 14 shows the protein band pattern.

*Forward run at pH 8.9 and reverse run at pH 4.3:* In one chamber the gel and the buffer composition was that of pH 8.9 system. In the other chamber the gel and buffer composition was that of pH 4.3 system. Broth sample (5 mg protein) was loaded in the gel of each chamber. The band patterns of the stained gels is shown in figure 15.

## **Discussion**

We report here a dual purpose simple and leakproof apparatus for vertical PA or agarose gel preparation and for the subsequent electrophoresis. Slab gels, as well as gel rods can be cast and run in the apparatus. Gel slabs of various thickness (0.04 cm to 1 cm) can be made and thus the apparatus can be used for analytical as well as for



Figures 8-12. 8. Two normal size gel slabs run together, each  $8.5 \times 0.3 \times 8.5$  cm. (a) Protein markers (ferritin, myoglobin, serum albumin and trypsin inhibitor), each  $400 \mu\text{g}$ . Position of the tracking dye is shown with arrows; (b) Protein markers in three wells; 15, 30, 45  $\mu\text{g}$  of each marker was loaded. Electrophoresis was at 90 V, 25 mA for 3 h. 9. Three thin gel slabs together, each  $8.5 \times 0.04 \times 8.5$  cm. Protein marker mixture containing 30  $\mu\text{g}$  of each marker

preparative purpose. More than one gel can be run in the apparatus. Gel rod cassette eliminates the troublesome removal of gels from the tubes. This is especially convenient for the gels of 15% or higher PA concentrations and does not require any rimming or tube breaking.

Hydrostatic balance (Chrambach and Nguyen, 1979) is automatically adjusted due to the position of the two buffer chambers. Mechanical stress (Chrambach and Nguyen, 1979) on the gel is also avoided by *in situ* polymerization of the gel and subsequent electrophoresis in the same apparatus. Contact gel is reusable. The band patterns obtained in the device described are as good as those obtained in other models.

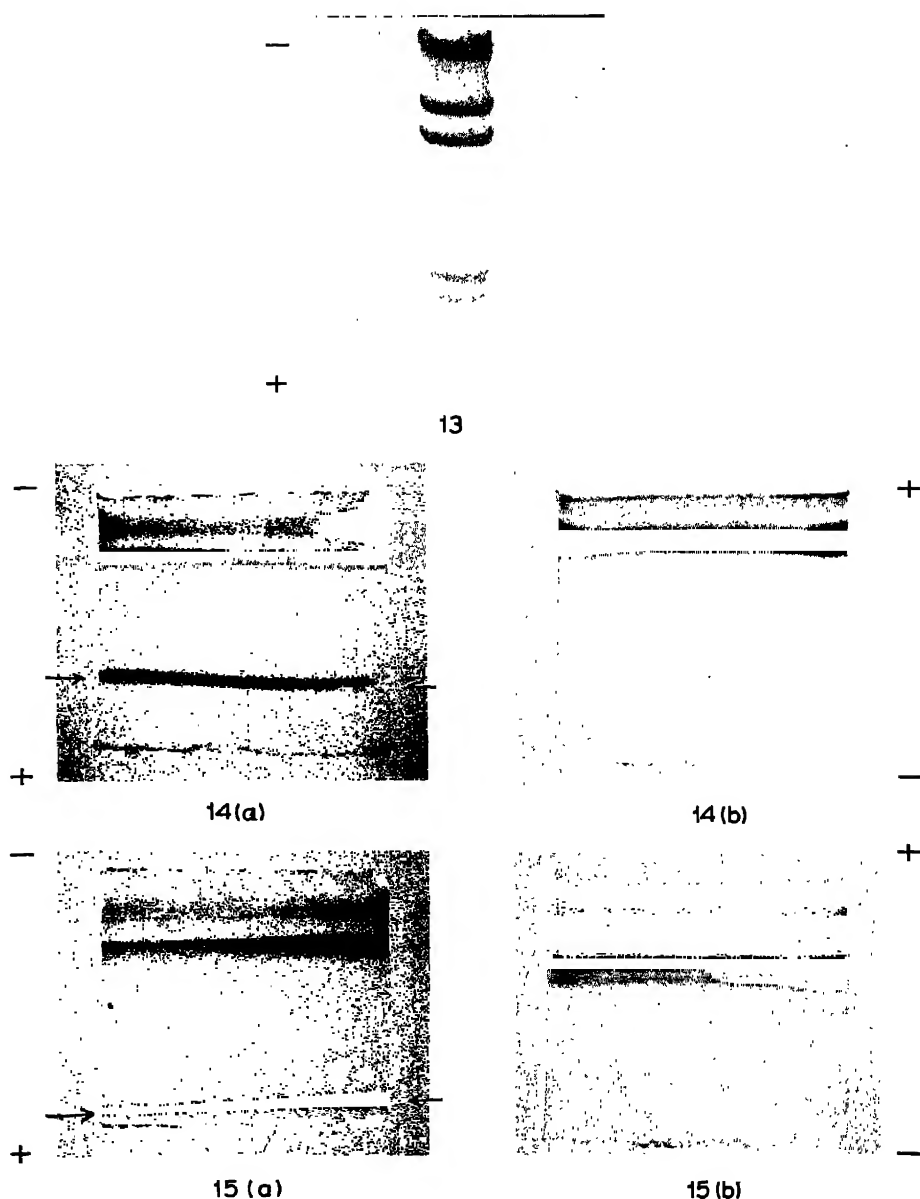
The apparatus shows some resemblance with the Pharmacia gel making apparatus (Pharmacia Fine Chemicals, Sweden, Gel Slab Casting Apparatus GSC-8). However, the introduction of one of the main vital modifications—the gap kept below the central partition in our apparatus has made a vast difference and made the unit a 'two in one' unit *i.e.* the same apparatus is used for gel casting and subsequent electrophoresis also, for which M/s. Pharmacia had to develop a separate and costly electrophoresis apparatus. In our device the gel thickness can be varied from 0.4 mm to 10 mm, which is not possible in the Pharmacia electrophoresis apparatus. In the gel rod cassette of the present apparatus, gels of very high acrylamide concentration (30 to 40% acrylamide) can be cast and the gel rods after the run can be easily removed from the cassette by just opening the two halves of the cassette like a book. Removal of high concentration gel rods is not possible in the commercial models. High concentration gel rods can be used for electrophoresing small molecular weight proteins and peptides. The present apparatus has also other advantages as mentioned earlier in 'Introduction'.

The modified device (figure 7) can be used for a simultaneous forward and reverse polarity electrophoresis of a sample. Thus in a single run it will be possible to get a total protein pattern of a sample. In most of the reported models this facility is not available. This modified device can also be used for usual electrophoresis (unidirectional) as in our normal device (figure 2). However, the normal device is more compact, cheaper and easy to fabricate as compared to the modified design. Although simultaneous forward and reverse electrophoresis can be carried out only in the modified device, for routine runs the compact device is quite satisfactory.

Under normal experimental conditions the problem of heat dissipation—elimination of heat generated in the gels, is well taken care of in our device. Even during the preparative run the temperature rise in the bath buffer and in the gel proper, is not more than 3°C and 7°C respectively. In the case of labile proteins, runs can be carried out in a cold room. Since glass is a better conductor of heat than perspex, one surface of the wedge facing the cassette and the middle partition wall between chamber 'a' and 'b' are made of glass. When chamber 'a' is filled with bath buffer the hollow wedge also gets filled with it which cools the gel through the glass surface. Also the glass wall of middle partition offers cooling by the bath buffer of chamber 'b'.

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was loaded. Electrophoresis at 100 V, 12 mA for 2 h. 10. One thick gel, 8.5 × 1 × 8.5 cm; preparative scale. Marker mixture, 7 mg of each marker (total 28 mg protein). Electrophoresis at 150 V, 30 mA, 4.5 h. 11. Gel rods of 15% PA concentration using a gel rod cassette 40, 40, 20 and 20 µg of each marker. Electrophoresis at 110 V, 15 mA for 4.5 h. 12. Electrophoresis of trypsin in two normal gel slabs in a cold room. 2 mg of trypsin in each gel. Electrophoresis at 120 V, 25 mA for 3 h.



**Figures 13–15.** 13. DNA marker in 0.8% agarose gel, normal size gel 0.1  $\mu$ g of DNA marker loaded in a well. Electrophoresis at 60 V, 15 mA for 2.5 h. 14. Simultaneous forward and reverse electrophoresis of fungal broth. (a) Forward run at pH 8.9; (b) Reverse run at pH 8.9. Broth sample (5 mg protein) in the gel of each chamber. Electrophoresis at 120 V, 20 mA for 5 h. 15. Simultaneous forward and reverse electrophoresis of fungal broth (a) Forward run at pH 8.9; (b) Reverse run at pH 4.3. Broth sample (5 mg protein) in the gel of each chamber. Electrophoresis at 120 V, 20 mA for 3.5 h.

The present construction is very simple and thus allows reproduction in a poorly equipped workshop. The design will be of help to those who have only rather small resources.

It is possible that when very high current densities are used, as in a few cases, the present apparatus may not be able to solve the heat dissipation problem, unless some additional cooling arrangement is introduced. This problem can however be easily solved by sandwiching the gel cassettes or the gel between cooling plates as described by Tichy (1966).

The hollow wedge can also be used as one of the cooling plates.

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## Toxicological evaluation of karaya gum; acute and subacute oral toxicity in rats

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**Abstract.** Male and female albino rats (Wistar strain) were given single and multiple doses of karaya gum suspended either in peanut oil or mixed with basal diet at different concentrations ranging from 0.5 to 8 g gum/kg body weight. The plant gum did not elicit any overt signs of toxicity or death in both sexes of rats.

Daily administration of karaya gum mixed with basal diet at different dose levels (0, 5, 20 and 40 g gum/kg diet) for a period of 90 days showed no adverse effects in male and female rats. The body weight, growth pattern, food and water intake were comparable with those of the normal rats. There were no significant biochemical, or morphological alterations in the vital organs of experimental animals.

**Keywords.** Karaya gum; acute and subacute study; rats.

### Introduction

Various kinds of gums of vegetable origin such as arabica, carob bean, tragacanth, guar and karaya have been used in medicine, cosmetics, food and textiles, etc. Emphasis has been placed in recent years on the increased use of plant gums in the preparation of food and pharmaceutical products to reduce the possible toxic effects of synthetic gums.

Karaya gum is the exudate of the plant, *Sterculia urens* and is popularly known as Sterculia in the west. Large quantities of karaya gum are obtained from *Sterculia urens* plants, grown in the forests of Madhya Pradesh, Maharashtra, Gujarat, Rajasthan and Orissa. Karaya gum of commercial grade is available as thick flakes or lumps of irregular shape and of ivory colour with a smell of acetic acid.

Karaya gum is used in several industries. In view of its low solubility in water and low cost it has a great export potential. Karaya gum has been used in printing and textile industry, in pharmaceutical and medicinal preparations such as cosmetics, lozenges, jellies, emulsions, lotions, sprays, pastes and laxatives and to control diarrhoea. It is also used as a binding and dressing spread in baking and dairy industries. Because of the properties of swelling and contraction in presence of moisture karaya gum powder is used in denture fixtures. The gum is used as an adhesive in ileostomy and colostomy appliances (Goligher and Pollard, 1972, 1973). The gum has also been in use as pulp binder, particularly in the manufacture of long fibres, light weight thin paper sheets.

Abbreviation used: RBC, Red blood cells.

The gum imparts smooth surface and strength to thin sheets of paper. The gum is used as an agent to accelerate the tannin action in the leather industry.

Additional information on cultivation and uses of karaya gum is available elsewhere (Wealth of India, 1976; Martindale, 1977). The present paper deals on the toxicological effect of karaya gum given to both sexes of rats through diet and oral intubation.

## Materials and methods

Male and female albino rats (Wistar strain) and of average body wt 156 g were kept separately in individual polypropylene cages with stainless steel hopper in air conditioned room (24°C) of the animal house under uniform animal husbandary conditions. The animals were fed basal diet (Hindusthan Lever Laboratory Animal Feed) and water *ad libitum*. The animals were acclimatized to temperature and lighting (12 h light/dark) conditions of the animal house.

### *Karaya gum*

Karaya gum was supplied by National Institute of Nutrition, Hyderabad. Clean flakes of gum was powdered into fine dust of 150 mesh.

### *Histopathology*

Sections of the above organs fixed in formal-saline solution were processed and cut at 6  $\mu$ m thickness and stained with haematoxylin-eosin.

### *Biochemical studies*

Freshly removed liver and brain separated from extraneous material in chilled saline medium were homogenised on 0.25 M ice-cold sucrose solution 10% w/v in a Potter Elvehjem type homogeniser. The homogenate was centrifuged at 700 *g* for 10 min to remove cell debris. Serum, red blood cells (RBC) (separated from the blood) and liver homogenate were used for the estimation of glutamic-oxaloacetic transaminase (EC 2.6.1.1), glutamic-pyruvic transaminase (EC 2.6.1.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) as per the colorimetric procedure of Wootton (1964). The estimation of protein was carried by the method of Lowry *et al.* (1951).

### *Haematological studies*

Blood samples of animals fed with single and multiple doses of karaya gum was analysed for haemoglobin content, total RBC and white blood cells (WBC) counts following the methods of Kolmer *et al.* (1957) and Wintrobe and Landsberg (1935) respectively.



### *Preparation of diet*

For each treatment finely powdered karaya gum was mixed with the basal diet. Fresh diet was prepared every week.

### *Acute toxicity study*

*Experiment No. 1:* Animals fasted overnight were given single and multiple oral doses of gum (0.5 to 5 g gum/kg body wt) suspended in peanut oil. Animals of the control group received only peanut oil with the same route.

*Experiment No. 2:* The fasted rats of both sexes were given basal diet alone or along with 0.5 to 8 g gum/kg body wt. The animals were fed diet containing karaya gum for 24 h after which normal basal diet was supplied to them.

All the animals of experiment No. 1 and 2 were observed for 15 days to record signs of toxicity and death if any. The LD<sub>50</sub> values and toxicity rating of karaya gum was determined (Weil, 1952 and Gleason *et al.*, 1969).

After the completion of observation period of 15 days all the animals were killed by decapitation. Necropsy was done to find gross pathological changes in the vital organs of the rats.

### *Subacute toxicity study*

Male and female rats 32 of each sex housed in individual cages and maintained under uniform animal husbandary conditions described above were given basal diet alone or mixed with 0, 5, 20 and 40 g of gum/kg of diet daily for a period of 90 days. After the completion of 90 days feeding study the animals were killed by decapitation. Blood was collected directly from the jugular vein into tubes containing double oxalate solution. The liver, kidney, adrenal, lung, brain, pituitary, spleen, pancreas, heart, testes, epididymis, ovary, uterus, cervix and vagina were removed and weighed individually. The organ/body weight ratio was calculated (Fisher, 1950).

## **Results**

The results of acute study of karaya gum given to both sexes of rats at different concentrations either suspended in peanut oil or mixed with basal diet could not induce any kind of clinical sign of toxicity or death in experimental animals.

### *Subacute study*

While animals of both sexes given 0, 5 and 20 g gum/kg of diet consumed the food normally, those dosed with 40 g gum/kg diet initially exhibited a kind of repellency or non-palatability to the mixed food. This repellency however, soon disappeared and the animals were acclimatized to this diet. The body weight, growth pattern, rate of food

and water intake of the animals given different levels of karaya gum was thus comparable with those of the normal rats (tables 1-3). The organ body wt ratio of animals exposed to the gum did not indicate any significant changes.

**Histopathology:** Autopsy of treated animals after 90 days exposure revealed no significant changes in the vital organs. Microscopic observation of liver, kidney, adrenal, spleen, brain, gastrointestinal tract, testes, epididymis, ovary, uterus, cervix and vagina of rats fed with different concentration of karaya gum for a period of 90 days also did not suggest any significant tissue damage and were comparable with those of control animals.

**Table 1.** Body wt\* (g) of male and female albino rats exposed to karaya gum for 13 weeks.

Dosage (g/kg/D**)	Weeks													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>Male</i>														
0	155	160	165	168	172	175	178	180	185	188	190	198	200	205
5	153	160	168	170	170	175	175	180	180	178	182	185	190	198
20	157	159	157	158	160	166	168	170	175	178	180	184	188	195
40	157	158	158	160	165	162	160	165	168	170	175	178	182	188
<i>Female</i>														
0	154	154	158	160	162	165	170	178	180	182	185	190	195	202
5	151	150	152	155	160	162	165	170	175	178	180	185	190	194
20	156	157	156	159	160	164	168	172	175	180	182	185	188	192
40	153	153	155	155	157	158	160	168	168	170	175	180	185	190

\* Average value of 16 animals in each treatment.

\*\* g of karaya gum/kg of diet.

**Table 2:** Food (g/day)\* intake of male and female albino rats exposed to karaya gum for 13 weeks.

Dosage (g/kg/D**)	Weeks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>Male</i>													
0	15.2	15.8	13.2	13.3	14.4	14.8	12.9	12.4	13.0	13.4	13.8	13.7	12.4
5	15.5	14.6	12.9	14.4	13.4	13.5	13.8	13.0	12.3	14.4	12.9	13.6	13.8
20	12.9	13.8	11.2	15.0	12.5	13.9	13.2	13.4	12.6	13.5	13.0	14.0	13.6
40	13.9	13.5	12.6	13.9	13.02	13.4	12.9	13.4	12.8	11.7	12.3	12.9	13.0
<i>Female</i>													
0	11.4	11.8	12.3	13.8	11.9	12.7	12.3	12.4	12.6	11.8	12.3	12.0	12.7
5	12.4	13.5	13.5	14.3	13.5	12.1	12.4	14.8	14.6	13.6	13.0	14.5	14.4
20	12.6	13.8	12.5	14.0	12.9	13.0	13.7	12.4	13.8	12.7	13.6	14.3	14.0
40	12.4	13.6	13.9	13.8	12.9	13.4	13.4	13.0	12.8	14.4	11.3	12.6	12.4

\* Average value of 16 animals in each treatment.

\*\* g karaya gum/kg of diet.

**Table 3.** Water\* (ml/day) intake of male and female albino rats exposed to karaya gum for 13 weeks.

Dosage (g/kg/D)**	Weeks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>Male</i>													
0	30.41	26.87	27.22	28.57	28.28	29.22	27.87	28.64	28.45	26.85	26.85	27.85	30.28
5	32.12	16.10	26.89	27.17	20.01	28.64	28.32	27.21	27.14	27.87	27.22	28.30	28.57
20	28.59	25.80	27.16	26.89	27.14	28.32	27.85	27.87	26.88	27.14	28.57	17.18	27.89
40	27.94	27.14	27.87	26.73	27.12	27.14	28.28	27.46	27.14	27.87	29.30	28.64	28.57
<i>Female</i>													
0	28.57	28.28	27.85	28.28	20.00	29.28	28.30	25.02	28.30	28.57	26.82	27.14	28.88
5	28.28	27.85	28.28	28.42	29.28	28.85	28.37	25.74	26.60	28.86	27.46	27.85	29.51
20	28.00	29.28	28.71	28.57	26.85	25.57	28.44	26.88	27.17	29.29	26.66	26.46	28.72
40	28.42	29.14	28.57	27.85	25.14	26.57	28.60	27.46	25.09	29.57	25.54	25.44	28.57

\* Average value of 16 animals in each treatment.

\*\* g karaya gum/g of diet.

**Biochemical studies:** The results of the activity of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and alkaline phosphatase of treated and control rats are shown in table 4. An increase in serum glutamic-oxaloacetic transaminase ( $P < 0.02$ ) and protein ( $P < 0.05$ ) and the liver enzyme ( $P < 0.01$ ) was noticed in male rats fed with 10 g gum/kg of diet. However female rats exposed to the high levels of gum showed a slight decrease in the liver glutamic-oxaloacetic transaminase ( $P < 0.05$ ) but at 10 g gum/kg of diet level the females indicated a significant increase in serum glutamic-oxaloacetic transaminase ( $P < 0.01$ ) activity. There was no other significant biochemical change in any of the dose levels of karaya gum in either sex of albino rats.

**Haematological studies:** Blood analysis of male and female animals after single and repeated feeding of karaya gum did not suggest any kind of haematological changes.

## Discussion

Karaya gum has been evaluated for the safety of the product by different workers using several species of animals and in man (Ivy and Isaacs, 1938; Hoelzel *et al.*, 1941; Carlson and Hoelzel, 1948; Figley, 1950; Holbrook, 1951; Wisconsin Alumni Res. Foundation Lab. 1964). It has been observed by FAO and WHO expert committee (1974) that the available toxicological information on karaya gum is wanting and require further study.

Animals given karaya gum either mixed with basal diet or suspended in peanut oil showed no ill effects. All the animals tolerated the maximum test doses of the karaya gum. Since there were no signs of toxicity or mortality of animals at the highest dose of 5 and 8 g of gum/kg of body wt, no LD<sub>50</sub> values of the gum could be determined.

Animals given repeated oral doses of karaya gum mixed with diet for 90 days also did not suggest any ill effect of the gum. The growth pattern, food and water intake of

**Table 4.** Biochemical changes in male and female albino rats exposed to karaya gum for 13 weeks.

Dosage (g/kg/D*)	Male				Female			
	0	5	20	40	0	5	20	40
<i>Alk. phos.</i>								
Liver	0.99 ± 0.13 0.09 ± 0.002	0.99 ± 0.04 0.10 ± 0.01	0.71 ± 0.12 0.09 ± 0.01	0.52 ± 0.14 0.08 ± 0.01	0.98 ± 0.22 0.12 ± 0.10	0.77 ± 0.11 0.08 ± 0.01	0.65 ± 0.02 0.12 ± 0.01	0.54 ± 0.12 0.07 ± 0.01
<i>GOT</i>								
Liver	15.56 ± 1.11 0.095 ± 0.005	17.02 ± 1.30 0.105 ± 0.005	19.52 ± 2.00 0.105 ± 0.002	20.72 <sup>b</sup> ± 0.13 0.116 <sup>c</sup> ± 0.002	18.15 ± 1.13 0.098 ± 0.005	20.69 ± 1.23 0.15 <sup>a</sup> ± 0.005	14.65 ± 1.54 0.10 ± 0.003	14.00 <sup>d</sup> ± 1.06 0.099 ± 0.09
<i>GPT</i>								
Liver	36.39 ± 1.28 0.28 ± 0.13	40.55 ± 1.15 0.24 ± 0.01	39.10 ± 0.82 0.37 ± 0.01	27.33 ± 1.33 0.21 ± 0.01	32.26 ± 1.36 0.30 ± 0.02	34.99 ± 0.72 0.29 ± 0.08	36.36 ± 1.6 0.38 ± 0.01	30.14 ± 1.43 0.31 ± 0.07
<i>Protein</i>								
Liver	126.78 ± 7.9 74.09 ± 3.3	137.5 ± 3.42 72.32 ± 3.4	137.5 ± 11.8 72.77 ± 3.9	140.71 ± 3.57 85.53 <sup>d</sup> ± 2.25	130.07 ± 16.95 70.17 ± 4.5	126.78 ± 7.9 73.21 ± 3.1	114.28 ± 5.9 75.18 ± 6.4	150.59 ± 12.25 76.35 ± 5.31

\* g of karaya gum/kg of diet.

Alk. phos.—Alkaline phosphatase ( $\mu$ mol phenol. lib/min/g or ml); GOT—glutamic oxaloacetic transaminase, GPT—glutamic pyruvic transaminase ( $\mu$ mol/min/g or ml); protein—(mg/g or ml).<sup>a</sup>  $P < 0.001$ ; <sup>b</sup>  $P < 0.01$ ; <sup>c</sup>  $P < 0.02$ ; <sup>d</sup>  $P < 0.05$ .

animals were comparable with those of the normal rats. The rise in the serum protein and also of liver glutamic-oxaloacetic transaminase activity after a high dose of karaya gum in male rats were of marginal significance. Absence of clinical signs of toxicity, morphological changes in vital organs, biochemical and haematological alterations in gum treated animals suggest that the plant gum offers no potential ill effect to animal. Similar observations have been made in rats given 10 and 20% karaya gum for a prolonged period (Hoelzel *et al.*, 1941; Carlson and Hoelzel, 1948). There was no untoward effect in the experimental animals given 8 g gum/kg body wt after an acute exposure while the subacute study indicated a tolerance limit of 4% of gum in diet. These observations are in confirmity with the reports of Taupin and Anderson (1982) who recorded the untoward effect of the gum as 5% (w/v).

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## Purification and characterization of chymotrypsin inhibitors from marine turtle egg white

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**Abstract.** The egg white of marine turtle (*Caretta caretta* Linn.) contains two chymotrypsin inhibitors and one trypsin inhibitor. The two chymotrypsin inhibitors were purified to homogeneity, as judged by ion-exchange chromatography, polyacrylamide gel and sodium dodecyl sulphate-gel electrophoresis, isoelectric focusing, immunochemical tests and sedimentation in the ultracentrifuge. Their sedimentation coefficient values were independent of protein concentration. Their amino acid composition was similar, and contained seven disulphide bonds, and methionine and carbohydrate moiety were absent. Each inhibitor consisted of a single polypeptide chain of 117 amino acids. The average molecular weight of each inhibitor, calculated from sedimentation and diffusion coefficient values, amino acid composition and sodium dodecyl sulphate-gel electrophoresis was 13000. Both the inhibitors were stable over the pH range of 2-11. They inhibited  $\alpha$ -chymotrypsin by forming enzyme-inhibitor complexes at a molar ratio of unity. The dissociation constant of each complex was  $1.06 \times 10^{-10}$  M. Both the inhibitors were indistinguishable in their physical, chemical and inhibitory properties except for their isoelectric points which were pH 5.23 for inhibitor A and pH 6.0 for inhibitor B. Chemical modification of all amino groups with trinitrobenzene sulphonate had no effect on their inhibitory activity.

**Keywords.** Protease inhibitor; chymotrypsin; turtle egg white.

### Introduction

In contrast to the extensive studies with protease inhibitors from avian egg whites (Laskowski and Sealock, 1971; Lin and Feeney, 1972), very little information is available on inhibitors from reptilian egg whites. Recently the purification and characterization of an acidic trypsin inhibitor from tortoise egg white were reported by Lay *et al.* (1982), and the primary structure of a basic trypsin inhibitor from Red Sea turtle egg white by Kato and Tominaja (1979).

In a previous communication (Ray *et al.*, 1982) we reported the presence of anti-chymotryptic activity in the egg white of marine turtle (*Caretta caretta* Linn.). In the present paper we describe the purification and properties of two chymotrypsin inhibitors obtained from the same source. Unlike avian ovomucoids and ovoidinhibitors, the inhibitors from turtle egg white had lower molecular weight and were devoid of a carbohydrate moiety.

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Abbreviations used: CM, Carboxymethyl; SDS, sodium dodecyl sulphate.

## Materials and methods

Eggs of freshly killed turtles (*Caretta caretta* Linn.) were collected from local markets.

Bovine  $\alpha$ -chymotrypsin (thrice crystallized) and trypsin (twice crystallized) were purchased from Worthington Biochemical Corporation, New Jersey, USA; *p*-tosyl-L-arginine methyl ester, N-acetyl-L-tyrosine ethyl ester and *p*-nitrophenyl acetate from Sigma Chemical Company, St. Louis, Missouri, USA; Sephadex G-50 (fine) and Carboxymethyl (CM) Sephadex C-50 from Pharmacia Fine Chemicals, Uppsala, Sweden; ampholine (pH 5–8) from LKB Produkter AB, Stockholm, Sweden. Other reagents were commercial preparation of the highest purity available.

### Purification of chymotrypsin inhibitors

**Step 1.** Egg whites from a single turtle (*C. caretta*) were filtered through cheese cloth, and the filtrate was treated with solid  $(\text{NH}_4)_2\text{SO}_4$  to 85% saturation. The precipitate was collected by centrifugation and dissolved in a minimum volume of water.

**Step 2.** The resulting solution was applied to a column (3.5  $\times$  75 cm, bed volume 720 ml) of Sephadex G-50 (fine) equilibrated with 0.1 M sodium acetate buffer, pH 4.2. Elution was carried out at room temperature with the same buffer. Active fractions containing both chymotrypsin and trypsin inhibitors were pooled and concentrated by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  (85% saturation). The precipitate was dissolved in and dialysed against 0.1 M sodium acetate buffer, pH 5.0 in the cold.

**Step 3.** The dialysed solution was applied to a column (2.5  $\times$  23 cm, bed volume 110 ml) of CM-Sephadex C-50 equilibrated with 0.1 M sodium acetate buffer, pH 5.0 and a linear gradient from 0 to 0.3 M NaCl in the equilibration buffer was used. First two active fractions designated as inhibitors *A* and *B* were separately pooled and rechromatographed under the same condition. They were dialysed exhaustively against water and freeze-dried.

### Protein estimation

Protein concentrations in egg whites and in fractions obtained in the early stages of purification were estimated according to Lowry *et al.* (1951) using bovine serum albumin as a standard. The concentrations of both inhibitors *A* and *B* were determined from their absorbance at 280 nm using a value of  $E_{1\%}^{1\text{cm}} = 11.5$  determined experimentally.  $\alpha$ -Chymotrypsin concentration was estimated by active site titration with *p*-nitrophenyl acetate (Kézdy and Kaiser, 1970).

### Assay of enzymic and inhibitory activities

$\alpha$ -Chymotrypsin and trypsin activities were determined at pH 7.5 and 27°C in the absence and presence of inhibitor by the pH-stat method (Walsch and Wilcox, 1970) using N-acetyl-L-tyrosine ethyl ester and *p*-tosyl-L-arginine methyl ester, as described earlier (Ray *et al.*, 1982). The inhibitory activity was calculated as the difference between enzyme activity in the absence and presence of inhibitor. One unit of inhibitory activity is expressed as a mg enzyme inhibited by the inhibitor. Specific activity is expressed as units of inhibitory activity per mg inhibitor.

### *Polyacrylamide gel electrophoresis*

Disc polyacrylamide gel electrophoresis was performed at pH 4.5 by the method of Reisfeld *et al.* (1962). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (1969).

### *Immunodiffusion and immunoelectrophoresis*

These were done according to Hammarstrom and Kabat (1969) using rabbit antiserum to purified inhibitor B.

### *Isoelectric focusing*

This was performed according to Vesterberg and Svenson (1966) in an LKB 8100 electrofocusing column using 1% ampholine in the pH range of 5–8. The sample was allowed to focus at 600 V for 72 h at 4°C. After focusing, fractions of 1.9 ml were collected, analysed for pH and absorbance at 280 nm and assayed for inhibitory activity.

### *Ultracentrifugation*

For the determination of sedimentation coefficients and diffusion coefficients of inhibitors, ultracentrifugal analyses were carried out with a Spinco Model E analytical ultracentrifuge at 59780 revolutions/min and 8766 revolutions/min respectively, as described previously (Ray *et al.*, 1982).

Partial specific volumes were calculated from the amino acid composition according to Cohn and Edsall (1943) and the value of 0.705 ml/g was obtained for both inhibitors A and B.

### *Amino acid analyses*

These were performed according to Spackman *et al.* (1958) on a Beckman Multichrom amino acid analyser. Samples were hydrolysed with 6 M HCl at 110°C for 24, 48 and 72 h under vacuum. The total cysteine content was determined as cysteic acid after performic acid oxidation (Hirs, 1967). Tryptophan content was determined after hydrolysis with *p*-toluenesulphonic acid in the presence of 3-(2-amino ethyl) indole (Liu and Chang, 1971).

### *Modification of free amino groups*

This was performed by reaction with 2, 4, 6-trinitrobenzene sulphonic acid at pH 8.5 according to the method of Habeeb (1966).

### *Check for carbohydrate content*

Attempts were made to measure neutral sugar by phenol-H<sub>2</sub>SO<sub>4</sub> method (Dubois *et al.*, 1956) and amino sugar by a modification of Elson-Morgan reaction (Davidson, 1966) using glucose and glucosamine, respectively as standards.

## Results and discussion

### Purification

Turtle egg white contained two chymotrypsin inhibitors and one trypsin inhibitor. Their separation and purification were achieved by gel filtration through Sephadex G-50 followed by ion-exchange chromatography on CM-Sephadex C-50 (figure 1). Both chymotrypsin inhibitors *A* and *B* on rechromatography gave single peaks with constant inhibitory activity across the peaks, indicating that preparations were probably homogeneous. A summary of the purification procedure is given in table 1. The yield of inhibitor *A* was about one-third of that of inhibitor *B*.

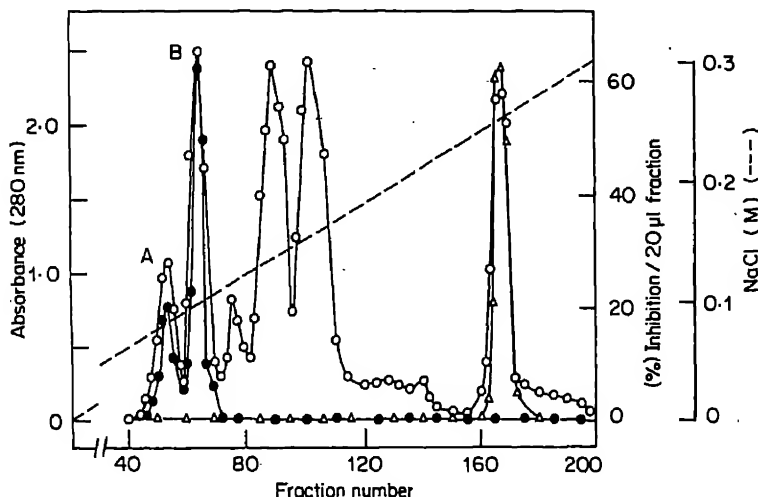


Figure 1. CM-Sephadex C-50 chromatography of active fraction from the gel filtration of turtle egg white. The column ( $2.5 \times 23$  cm, bed volume 110 ml) was equilibrated with 0.1 M sodium acetate buffer, pH 5.0. About 900 mg protein was applied to the column. Elution was performed using a linear gradient of 0–0.3 M NaCl in the same buffer. Fractions of 5.0 ml were collected at a flow rate of 30 ml per h. (○), absorbance at 280 nm; (●), chymotrypsin inhibition; (Δ), trypsin inhibition.

### Homogeneity

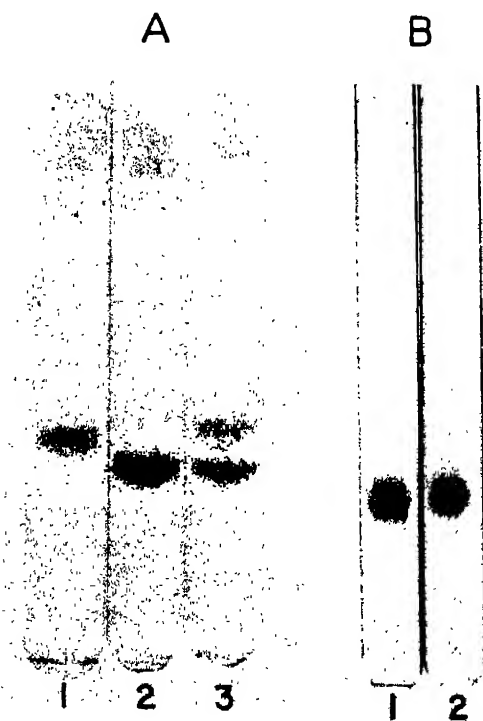
Both inhibitors *A* and *B* were homogeneous on polyacrylamide gel electrophoresis at pH 4.5 and on SDS-gel electrophoresis at pH 7.0 in the presence of 2-mercaptoethanol, as shown in figure 2. Isoelectric focusing of both inhibitors yielded single peaks with constant inhibitory activity across the peaks with isoelectric points at pH 5.23 for inhibitor *A*, and at pH 6.0 for inhibitor *B*.

Ultracentrifugal analysis of both inhibitors also showed single symmetrical peaks at different protein concentrations (0.5–1.4%) with sedimentation coefficient ( $S_{20,w}$ ) values of 1.63S for inhibitor *A* and 1.66S for inhibitor *B*. Their sedimentation coefficients were essentially independent of protein concentration.

**Table 1.** Summary of the purification of chymotrypsin inhibitors from turtle egg white.

Step	Chymotrypsin inhibitory activity (units)	Specific activity (units/mg)	Recovery of activity (%)	Purification
Egg white	686	0.07	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	624	0.08	91	1
Gel filtration through Sephadex G-50	368	0.41	54	6
Chromatography on CM-Sephadex G-50				
Inhibitor <i>A</i>	80	1.96	12	30
Inhibitor <i>B</i>	200	1.74	29	24
Rechromatography on CM-Sephadex C-50				
Inhibitor <i>A</i>	60	2.00	9	30
Inhibitor <i>B</i>	170	1.96	26	29

Inhibitor activity and protein content were determined as described under Materials and methods.



**Figure 2.** Polyacrylamide gel electrophoresis of chymotrypsin inhibitors of turtle egg white on (A) 10% gel at pH 4.5 and (B) 7.5% gel containing 0.1% SDS at pH 7.0. 1, inhibitor *A*; 2, inhibitor *B*; 3, a mixture of inhibitors *A* and *B*.

The homogeneity of both inhibitors was also shown by immunodiffusion and immunoelectrophoresis where each inhibitor gave a single precipitin line of identity (figure 3). The cross-reactivity of inhibitor *A* against rabbit anti-inhibitor *B* serum indicates the immunological identity of the two inhibitors.

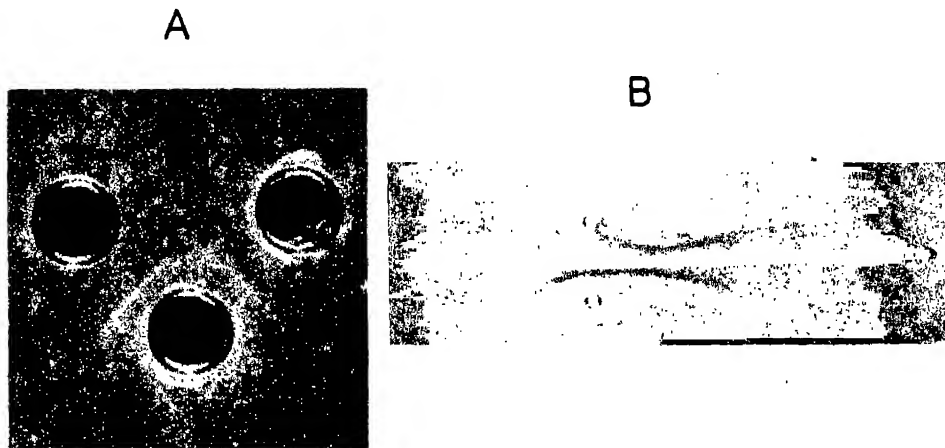


Figure 3. A. Immunodiffusion of chymotrypsin inhibitors of turtle egg white. The central well contained the antiserum to inhibitor *B* and left and right wells contained inhibitors *A* and *B* respectively. B. Immunoelectrophoresis of chymotrypsin inhibitors from turtle egg white. The central slot contained the antiserum to inhibitor *B* and the upper and lower slots contained inhibitors *A* and *B* respectively. Electrophoresis was done in 0.05 M veronal buffer, pH 9.0 at 5v/cm and 25°C.

### Molecular weight

The molecular weight of both the inhibitors was estimated to be 12900 by SDS-polyacrylamide gel electrophoresis. The diffusion coefficients of inhibitors *A* and *B* calculated from the boundary spreading in the ultracentrifuge (Baldwin, 1957) were  $10.3 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ , and  $10.6 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$  respectively. From the sedimentation-diffusion data using the Svedberg equation the molecular weights were found to be 13000 for inhibitor *A* and 12900 for inhibitor *B*. Amino acid compositions gave a molecular weight of 13100 for both the inhibitors. From the above results the average molecular weight of both the inhibitors was calculated as 13000, which was similar to those of tortoise and turtle egg white trypsin inhibitors (Ray *et al.*, 1982). In contrast, the molecular weights of avian ovomucoids and ovoinhibitor (Lin and Feeney, 1972) were reported as 28000 and 49000 respectively.

### Amino acid composition

Table 2 shows the amino acid compositions of inhibitors *A* and *B*. It may be noted that both the inhibitors have identical number of individual amino acid residues. Their amino acid composition is markedly different from the compositions of tortoise and

**Table 2.** Amino acid compositions of chymotrypsin inhibitors *A* and *B* of turtle egg white.

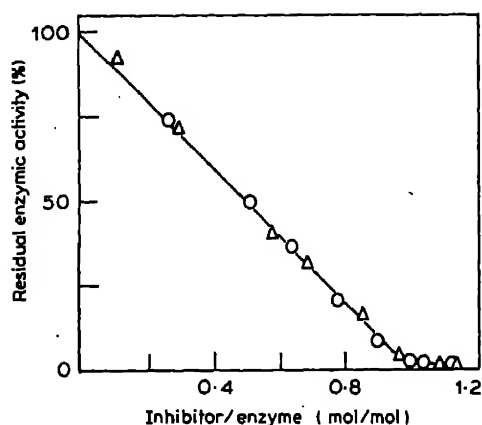
Amino acid	Residues/mol		Integer
	Inhibitor <i>A</i>	Inhibitor <i>B</i>	
Lys	9.1	8.6	9
His	1.8	1.9	2
Arg	2.0	2.0	2
Asp	10.8	10.7	11
Thr <sup>a</sup>	6.7	6.8	7
Ser <sup>a</sup>	7.8	7.9	8
Glu	15.7	16.0	16
Pro	6.3	5.7	6
Gly	12.5	12.8	13
Ala	3.0	3.0	3
$\frac{1}{2}$ Cys <sup>b</sup>	14.2	13.9	14
Val <sup>c</sup>	2.7	2.9	3
Met	0	0	0
Ile	1.7	1.8	2
Leu	5.6	5.8	6
Tyr	5.1	4.8	5
Phe	8.6	8.6	9
Trp <sup>d</sup>	0.9	0.9	1

<sup>a</sup>Values obtained by extrapolation to zero hydrolysis time.<sup>b</sup>Determined as cysteic acid after performic acid oxidation.<sup>c</sup>Values are yields from 72 h hydrolysates.<sup>d</sup>Determined after hydrolysis with *p*-toluenesulphonic acid.

turtle egg white trypsin inhibitors reported earlier (Ray *et al.*, 1982). Both chymotrypsin inhibitors *A* and *B* contained 14 half-cystine residues, and one tryptophan residue, and lacked methionine residue. Free sulphhydryl groups could not be detected by Ellman's reaction (Ellman, 1959) even in the presence of 8 M urea. Since SDS-gel electrophoresis in the presence and absence of 2-mercaptoethanol showed the presence of a single polypeptide chain of the same mobility, it was concluded that all of the half-cystine residues were present in both the inhibitors as disulphide bonds. No N-terminal amino acid residue was found in the performic acid oxidized derivatives of both the inhibitors, as judged by dansyl chloride method (Gray, 1967). Like tortoise and turtle egg white trypsin inhibitors, they lacked carbohydrate moiety. In this respect they differ from avian ovomucoids and ovoinhibitors which are glycoproteins.

#### *Inhibitor properties*

Both inhibitors *A* and *B* inhibited bovine  $\alpha$ -chymotrypsin, but not trypsin. The inhibition of chymotrypsin at pH 7.5 by increasing amounts of inhibitors *A* and *B* is shown in figure 4. It was noted that the interaction of chymotrypsin with inhibitor *A* was identical to that with inhibitor *B*. The titration curve was linear up to 98% inhibition and extrapolation of the data indicated that one mol of the inhibitor reacted



**Figure 4.** Inhibition of  $\alpha$ -chymotrypsin by turtle egg white inhibitor *A* (○) and inhibitor *B* (Δ). Assays were performed on N-acetyl-L-tyrosine ethyl ester at pH 7.5 and 27°C after 2 min preincubation of enzyme and inhibitor.

with one mol of  $\alpha$ -chymotrypsin to form a 1:1 inhibitor-enzyme complex. The dissociation constant of the complex calculated according to Green and Work (1953) was found to be  $1.06 \times 10^{-10}$  M for each inhibitor.

Both the inhibitors *A* and *B* were found to be stable over the pH range of 2–11. No inhibitory activity was lost when each inhibitor was heated to 100°C at neutral pH for 5 h. The high stability was probably due to the presence of seven disulphide bonds, small size and compact nature of these molecules.

Reaction with trinitrobenzene sulphonate led to the complete modification of free amino groups present in the inhibitors, but their inhibitory activities were not affected. This suggested that the amino groups were probably not necessary for the inhibition of chymotrypsin.

Results presented in this study indicated clearly that chymotrypsin inhibitors *A* and *B* of turtle egg white were very similar in their physical, chemical and inhibitory properties, except that inhibitor *A* was more acidic in nature than inhibitor *B*. This difference was reflected in their elution profiles on ion-exchange chromatography, polyacrylamide gel electrophoresis and isoelectric focusing. Since both the inhibitors contained identical number of individual acidic amino acids and basic amino acids, it was apparent that inhibitor *A* had lesser amide content than inhibitor *B*. These two inhibitors might be considered as iso-inhibitors.

### Acknowledgements

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## Evaluation of fractionated *Wuchereria bancrofti* microfilarial excretory-secretory antigens for diagnosis of bancroftian filariasis by enzyme linked immunosorbent assay

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**Abstract.** The *Wuchereria bancrofti* microfilarial excretory-secretory antigens were fractionated into ES1, ES2, ES3 and ES4 by ultra-membrane filtration and evaluated for their diagnostic utility by enzyme linked immunosorbent assay. Three of the four fractions showed antigenic activity (ES2, ES3 and ES4). The antigen fractions ES2 and ES4 were highly active in the detection of filarial IgM antibody in clinical filariasis and microfilaraemia respectively. The chemical characterization of the ES2 and ES4 antigen fractions showed that they were glycoproteins.

**Keywords.** Microfilarial excretory-secretory antigen; ultramembrane filtration; enzyme linked immunosorbent assay.

### Introduction

Excretory-secretory (ES) antigens were highly sensitive and specific tools for the diagnosis of parasite diseases such as chagas disease (Tarrant *et al.*, 1965), toxocoriosis (de Savigny *et al.*, 1977) and onchocerciasis (Schiller *et al.*, 1980). Studies from this laboratory have shown that the *Wuchereria bancrofti* microfilarial (mf) ES antigens are highly sensitive in detecting antibody in filarial sera as well as in filter paper blood samples (Kharat *et al.*, 1982; Malhotra *et al.*, 1982). However mf ES antigen from culture fluid could not distinguish active infection from clinical filariasis in enzyme linked immunosorbent assay, using either mixed or specific anti-immunoglobulin-enzyme conjugates. Separation and characterization of different antigen fractions from the whole antigen has proved to be a meaningful approach in obtaining an active fraction with the required specificities (Sawada *et al.*, 1969; Marcoullis *et al.*, 1978; Kaliraj *et al.*, 1982). This communication presents the fractionation and characterization of mf ES antigens and studies on their diagnostic utility for detecting human filariasis.

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Abbreviations used: ES, Excretory-secretory; ELISA, enzyme-linked immunosorbent assay; mf, microfilarial; PBS/T, 0.01 M Sodium phosphate buffer saline pH 7.2, containing 0.05% Tween 20; IgG + M + A, antihuman immunoglobulins; GMRT, geometric mean of the reciprocal of antibody titre.

## Materials and methods

### *Microfilarial excretory-secretory antigens*

The mf ES antigens were prepared as described by Kharat *et al.* (1982). *W. bancrofti* mf were separated from microfilaraemia blood samples by nucleopore membrane filtration and maintained for 15 days in medium 199 (3–4 thousand mf/ml medium) supplemented with organic acids and sugars of *Grace's medium* (Paul, 1975). The medium was changed after every 24 h and the culture fluid was centrifuged at 13,000 *g* for 15 min. The supernatant was collected and the protein was estimated by Lowry's method (Lowry *et al.*, 1951). Then it was stored at  $-20^{\circ}\text{C}$  until used, after addition of sodium azide to a final concentration of 0.1% as preservative.

### *Sera*

Human sera (32 samples), belonging to different groups *viz.*, normal subjects from endemic and non endemic regions (endemic and non endemic), filarial patients (microfilaraemia and clinical filariasis) were screened in this study. Filarial blood samples were collected from Sevagram and surrounding villages, which is an endemic area for nocturnally periodic form of *W. bancrofti*. Endemic normal samples were from healthy individuals living in Sevagram and its surrounding villages and having neither mf in their blood nor any clinical symptoms. The presence or absence of mf was confirmed by night blood (wet smear) examination. Non endemic normal blood samples were collected from students of this Institute coming from places like Chandigarh, Kashmir etc. where there is no filariasis immediately after their admission. Sera were separated from blood samples and stored at  $-20^{\circ}\text{C}$  after addition of sodium azide as preservative.

### *Fractionation of mf ES antigen*

The fractionation of mf ES antigen into different fractions by ultra membrane filtration was essentially as described by Nash *et al.* (1974) with *S. mansoni* homogenate and by Mok *et al.* (1977) with *Histoplasma capsulatum* antigen, except that Millipore ultrafiltration system (Millipore Corporation, Bedford, USA) was used in this study. One ml of culture fluid containing mf ES antigen was diluted to 20 ml with 0.05 M sodium phosphate buffer pH 7.2 and sequentially passed through CX-10 (NMWL 10,000), CX-30 (NMWL 30,000) immersible ultrafilters and PTHK 02510 (NMWL 100,000) pellicone ultra-filter membrane. At each stage of filtration, the filtrate was collected and the concentrate (0.1 ml) was diluted to 20 ml with the same buffer and refiltered. The process of dilution and refiltration was repeated twice and these filtrates were discarded. The concentrate was diluted to 20 ml with 0.05 M sodium phosphate buffer pH 7.2 for passing through the next filter in sequence. The filtrates collected first from CX-10, CX-30 and PTHK-02510 ultra filters were labelled as ES1, ES2 and ES3 fractions respectively and the concentrate on PTHK-02510 was diluted to 20 ml with 0.05 M sodium phosphate buffer pH 7.2 and labelled as ES4. All the four fractions were diluted 500 times with sodium carbonate buffer 0.06 M, pH 9.6 and used in indirect enzyme linked immunosorbent assay (ELISA) to assess their diagnostic utility.

### Characterization of ES2 and ES4 antigen fractions

**Treatment with enzymes:** Three ml each of diluted (50 times) mf ES antigen fractions ES2 and ES4 in 0.05 M sodium phosphate buffer, pH 7.2 were treated separately with an equal volume of the following enzymes (at final concentration, 50 µg/ml); chymotrypsin (Council of Scientific and Industrial Research Centre for Biochemicals, Delhi) in 0.05 M Tris-HCl buffer pH 7.4,  $\alpha$ -amylase (Fluka, Switzerland), in 0.05 M Sodium phosphate buffer, pH 7.2 and lipase (Scientific and Industrial Supplies Corporation, Bombay) in 0.05 M citrate buffer pH 6.0. The incubation of the antigen-enzyme mixture was at 37°C for 24 h as described by Rolfe and Finegold (1979), followed by centrifugation at 13000 *g* for 20 min at 4°C. The supernatant was collected and diluted (5 times) to the optimum concentration of each antigen fraction with 0.06 M sodium carbonate buffer, pH 9.6 and used in ELISA to see the effect of different enzymes on the antigen fraction.

**Treatment with sodium periodate:** The antigen fractions ES2 and ES4 were diluted 50 times with 0.05 M sodium phosphate buffer pH 7.2 and treated separately with an equal volume of 0.1 M sodium periodate and kept at 37°C for 24 h (Mok *et al.*, 1977). The mixtures were centrifuged and the supernatants were used in ELISA as described above.

**Heat inactivation:** The tubes containing diluted (50 times) ES2 and ES4 antigen fractions were kept in boiling water bath for 45 min, the fractions were then centrifuged and the supernatants were used in ELISA after diluting 10 times with 0.06 M sodium carbonate buffer pH 9.6.

In one control experiment, the antigen fractions (ES2 and ES4) were mixed with an equal volume of 0.05 M Tris-HCl buffer pH 7.4, kept at 37°C for 24 h and used in ELISA. In another control experiment each antigen fraction was treated separately with heat inactivated enzymes and after centrifugation (13000 *g* for 20 min), the supernatants were collected and used in ELISA.

### ELISA

Penicillinase Type I (5 mg) Sigma Chemical Co., Saint Louis, Missouri, USA with specific activity of 2100 units/mg protein was conjugated to 40 mg of antihuman immunoglobulins (anti IgG + M + A) or individual anti-IgG or anti IgM (Immunodiagnosics, New Delhi) by the single step method of Avrameas using glutaraldehyde (Avrameas, 1969). The substrate consisted of soluble starch (150 mg) in 27.5 ml of 0.2 M sodium phosphate buffer pH 7.0 containing 10.64 mg penicillin 'V' (Sigma Chemical Co., USA) and 65 µl of 0.08 M iodine in 3.2 M potassium iodide solution. The substrate was prepared fresh before use.

Micro ELISA was carried out as described by Kharat *et al.* (1982) in Cooke polyvinyl chloride microtitre U plates (No. 1-220-29). The optimally diluted mf ES, ES2 and ES4 antigen fractions in 0.06 M sodium carbonate buffer pH 9.6 were added to the wells and incubated at 37°C for 3 h. The wells were drained and the plate was further incubated with 200 µl of 3% bovine serum albumin in the same carbonate buffer at 37°C for 2 h. The plate was then washed 5 times with 0.01 M phosphate buffer saline pH 7.2 containing 0.05% Tween 20 (PBS/T). Hundred µl of diluted test sera (1:300) or serially

diluted (2 fold) test sera in PBS/T were added to the wells and incubated at 37°C for 3 h or at 4°C for overnight. After washing, 200 µl of 3 % bovine serum albumin in PBS/T was added to each well and incubated at 37°C for 2 h. After washing the plate again, 100 µl of optimally diluted conjugate in PBS/T (1:1600 for anti IgG + M + A penicillinase, 1:1600 for anti IgG penicillinase and 1:1000 for anti IgM penicillinase) was added and incubated at 37°C for 3 h. Following the final washing of the plate the immune reaction was revealed by adding 100 µl of the substrate solution to each well and incubating at 37°C for 30 min. The enzyme reaction was then terminated by adding 50 µl of 5 N HCl and the results were evaluated visually. The complete decolorization or decolorization with slight tinge of blue colour denoted the positive reaction while the negative reaction was confirmed by the persistence of blue colour. The antibody titres in the serum was the highest serum dilution showing decolorization with a slight tinge of residual blue colour.

## Results

*W. bancrofti* mf ES antigen from culture fluid was fractionated by membrane filtration into four fractions of different molecular weights namely ES1 ( $\leq 10,000$ ), ES2 (10,000–30,000), ES3 (30,000–100,000) and ES4 ( $\geq 100,000$ ). The antigen fractions ES2, ES3 and ES4 showed the presence of antibody in all of the 8 microfilaraemia, 8 clinical filarial and none of the 2 non-endemic and 6 endemic normal sera, when tested in indirect ELISA, using anti-IgG + M + A conjugate (table 1). Two sera from each group of microfilaraemia and clinical filariasis were screened for filarial IgG and IgM antibody titres using the three antigen fractions (ES2, ES3 and ES4). The results (table 2) revealed that the two antigen fractions ES2 and ES4 showed different sensitivities in determining the filarial IgM antibody in microfilaraemia and clinical filariasis. The mean values of reciprocal of filarial IgM antibody titres in microfilaraemia sera were 750 and 12000 with ES2 and ES4 antigens respectively, whereas clinical filarial sera showed mean values of reciprocal of filarial IgM antibody titres of 12000 and 300. No significant difference was observed between the two groups of sera using anti IgG conjugate.

Table 1. Detection of antibody in human filarial sera by indirect ELISA using mf ES antigen fractions and anti IgG + M + A-penicillinase conjugate.

Sera	No Exam.	Number showing positive reaction* employing			
		ES1	ES2	ES3	ES4
Nonendemic normal (mf - ve)	2	0	0	0	0
Endemic normal (mf - ve)	6	0	0	0	0
Microfilaraemia (mf + ve)	8	0	8	8	8
Clinical filariasis (mf - ve)	8	0	8	8	8

\* Sera showing positive reaction for filarial antibody at the dilution of 1:300.

**Table 2.** Reciprocal of IgM and IgG antibody titres in filarial sera using ES2, ES3 and ES4 antigen fractions.

Sera	Reciprocal of antibody titre (mean of 2 sera) employing					
	ES2		ES3		ES4	
	IgM	IgG	IgM	IgG	IgM	IgG
Microfilaraemia (mf + ve)	750	19200	4800	19200	12000	19200
Clinical filariasis (mf - ve)	12000	19200	1200	12000	300	12000

Table 3 shows the analysis of 12 microfilaraemia and 12 clinical filarial sera for filarial IgM antibody titres using ES2 and ES4 antigen fractions. Statistical analysis of the reciprocal of antibody titres was carried out using Mann-Whitney U test. ES2 antigen fraction showed higher geometric mean of reciprocal of antibody titre (GMRT) in clinical filariasis (7620) than in microfilaraemia (2138) and the difference was significant ( $P < 0.05$ ). ES4 antigen fraction showed higher GMRT in microfilaraemia (9600) than in clinical filariasis (1347) and the difference was found to be significant ( $P < 0.01$ ). The differences in the antibody titres with ES2 and ES4 antigens in microfilaraemia ( $P < 0.05$ ) as well as in clinical filariasis ( $P < 0.025$ ) were also found to be significant.

The ES2 and ES4 antigen fractions were found to be highly reactive in detecting IgM antibody from clinical filarial and microfilaraemia sera respectively and hence these sera were used to assess the reactivity of concerned antigen fraction after treatment with different agents. The ES2 and ES4 antigen fractions when treated with buffer alone showed filarial IgM antibody titres of 9600 and 18200 respectively (table 4). After chymotrypsin treatment or heating at 100°C the ES2 antigen showed an antibody titre

**Table 3.** Distribution of filarial IgM antibody titre in microfilaraemia and clinical filariasis using ES2 and ES4 antigen fractions.

Antigen fraction	Sera	No Exam.	Reciprocal of filarial IgM antibody				GMRT*
			300	1200	4800	19200	
ES2†	Microfilaraemia (mf + ve)	12	3	4	2	3	2138 <sup>a</sup>
	Clinical filariasis (mf - ve)	12	1	—	5	6	7620 <sup>b</sup>
ES4†	Microfilaraemia (mf + ve)	12	—	1	4	7	9600 <sup>c</sup>
	Clinical filariasis (mf - ve)	12	6	2	1	3	1347 <sup>d</sup>

† Sera used were same for both the antigens.

\* Geometric mean of reciprocal of antibody titres.

a vs. b ( $P < 0.05$ ); c vs. d ( $P < 0.01$ ); a vs. c ( $P < 0.05$ ) and b vs. d ( $P < 0.025$ )—statistically significant.

of 300 with pooled clinical filarial serum and ES4 antigen showed an antibody titre of 1200 with pooled microfilaraemia serum. When treated with sodium periodate or  $\alpha$ -amylase ES2 antigen showed antibody titre of 600 and ES4 antigen showed antibody titre of 300 with pooled clinical filarial and microfilaraemia sera respectively. However, there was no change in antibody titres in the control experiments where the ES2 and ES4 antigens were treated with heat inactivated enzymes. None of these treated and untreated antigen fractions showed reaction with non-endemic normal sera.

Table 4. Reciprocal of IgM antibody titre of pooled filarial sera with ES2 and ES4 antigens before and after treatment with different agents.

Treatment of antigen fraction with	Reciprocal of ELISA IgM antibody titre employing	
	ES2*	ES4*
Tris-HCl buffer	9600	18200
Sodium periodate	600	300
$\alpha$ -Amylase	600	300
Chymotrypsin	300	1200
Heat inactivation	300	1200
Lipase	9600	18200

\* Pooled clinical filarial serum and pooled microfilaraemia serum were used for ES2 and ES4 antigen fractions respectively.

## Discussion

*W. bancrofti* mf ES antigen from culture fluid was found to be highly sensitive when used in ELISA for the diagnosis of filariasis and only as little as 0.35 ng antigen per well was found to be sufficient (Kharat *et al.*, 1982). But no correlation was observed between antibody titres and disease status of the infection and mf ES antigen could not distinguish between microfilaraemia and clinical filariasis. This has necessitated the fractionation of mf ES antigen by membrane filtration, the method better suited for separation of minute amounts of ES antigens. Out of the four fractions obtained, three showed the antigenic activity. Nash *et al.* (1974) have sequentially passed the *S. mansoni* homogenate through Amicon Diaflo ultra filter membranes and found the antigen activity in only one fraction, having molecular weight of above 100,000. Mok *et al.* (1977) fractionated *H. capsulatum* yeast cell antigens by membrane filtration and observed the polydispersed nature of the antigens in several fractions.

Two of the three antigen fractions namely ES2 and ES4 showed different sensitivities while determining the filarial antibody titres in clinical filariasis and microfilaraemia (table 3). ES2 antigen detected elevated filarial IgM antibody (GMRT = 7620) in clinical filariasis than in microfilaraemia (GMRT = 2138) and ES4 in microfilaraemia (GMRT = 9600) than in clinical filariasis (GMRT = 1347). The differences were statistically significant. Similar observations were made with the two antigen fractions



mfS1b and mfS3e isolated from soluble antigens (mfS) of *W. bancrofti* microfilariae (Kaliraj *et al.*, 1982). It would be of interest to identify and characterize these antigens.

Studies have been carried out in many parasite infections to identify the chemical nature of the antigens (Nash *et al.*, 1974; Carlier *et al.*, 1978; Tanner, 1963). Treatment of mf ES antigen fractions ES2 and ES4 with lipase did not reduce their reactivity. The reactivity of both the antigen fractions was reduced as observed from reduction in ELISA antibody titres, after treating with chymotrypsin or heating at 100°C, due to the degradation or inactivation of the protein part of the antigens. The loss in reactivity of both the antigen fractions was also observed after treatment with sodium periodate or  $\alpha$ -amylase. This might be due to the destruction of the carbohydrate part of the antigens. These observations suggest that both the mf ES antigen fractions ES2 and ES4 might be glycoproteins.

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## Cyclic AMP transport across membrane vesicles of ultra-violet light irradiated *Escherichia coli*

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**Abstract.** Transport of cyclic AMP across *Escherichia coli* membrane was studied using membrane vesicles. Uptake of cyclic AMP was measured using normally oriented vesicles, whereas uptake in everted vesicles was taken as a measure of the efflux of cyclic AMP. Ultra-violet irradiation of the cells led to an inhibition of both uptake and efflux of cyclic AMP across the membrane. The presence of cyclic AMP in the growth medium prior to ultra-violet irradiation caused an enhancement of the uptake and efflux. The uptake and efflux of cyclic AMP were less in vesicles from glucose grown cells as compared to the uptake and efflux by the vesicles prepared from glycerol grown cells. Similarly both uptake and efflux of cyclic AMP were more in vesicles prepared from cells grown on glycerol or glucose in the presence of cyclic AMP than in vesicles from cells grown in absence of cyclic AMP. It is suggested that the number of cyclic AMP carrier molecules were reduced in cells under catabolite repression by glucose as well as by ultra-violet irradiation.

**Keywords.** cyclic AMP transport; membrane vesicles; ultra-violet irradiation; catabolite repression.

### Introduction

Ultra-violet (UV) irradiation of *Escherichia coli* B/r cells causes catabolite repression of  $\beta$ -galactosidase (Swenson, 1972) and L-arabinose isomerase (Bhattacharya, 1974). It was reported from this laboratory that intracellular cyclic AMP level was lowered when the cells were irradiated with UV light. Simultaneously, there was an increase in the level of the nucleotide in the suspending medium (Bhatnagar and Bhattacharya, 1982), suggesting that cyclic AMP is excreted from the cell into the suspending medium. Restoration of both intracellular and extracellular cyclic AMP levels to those in unirradiated cells following dark recovery (Bhatnagar and Bhattacharya, 1983) further supported this concept. Similar changes in the cyclic AMP level in cells under condition of catabolite repression by glucose is well known (Pastan and Adhya, 1976). It was therefore thought that catabolite repression may be due to changes in the transport properties of the membrane for cyclic AMP resulting in the changes in the nucleotide level. Transport of cyclic AMP could not be studied by using whole cells of *E. coli* because of nonspecific binding of the nucleotide with the cell wall material (Goldenbaum and Hall, 1979). Isolated membrane vesicles have proved to be particularly useful model system for the studies of transport of various substances (Kaback, 1970, 1974). For this reason, Goldenbaum and Hall (1979) used *E. coli*

membrane vesicles for studying the uptake and efflux of cyclic AMP. In order to compare the changes in cyclic AMP levels in cells following UV light irradiation with transport of cyclic AMP, membrane vesicles have been used as a tool in the present study to find out whether UV irradiation of *E. coli* cells causes any change in cyclic AMP transport across the membrane.

### Materials and methods

*E. coli* B/r (ORNL) was kindly supplied by Dr. P. A. Swenson of Oak Ridge National Laboratory, USA. Lysozyme and cyclic AMP were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. [ $^3\text{H}$ ]-Cyclic AMP (10.1 mCi/mmol) and [ $^{14}\text{C}$ ]-L-leucine (203 mCi/mmol) were obtained from Bhabha Atomic Research Centre, Bombay. Millipore filters (25 mm dia, 0.45  $\mu$  pore size) were purchased from Maxflow, India. All other chemicals were of analytical grades.

Membrane vesicles were prepared from exponentially growing culture of *E. coli* B/r by the lysozyme EDTA method of Kaback (1971). Vesicle preparations contained 3 to 5 mg of protein per ml as measured by the method of Lowry *et al.* (1951). Before studying cyclic AMP transport, each vesicle preparation was tested for its ability to actively transport [ $^{14}\text{C}$ ]-L-leucine according to the method of Lombardi and Kaback (1972). Vesicles having the capability of transporting L-leucine actively were only used to study the transport of cyclic AMP.

The uptake of cyclic AMP was measured following the procedure of Goldenbaum and Hall (1979). The reaction mixture (0.4 ml) contained 0.2 ml of membrane vesicles, 0.05 M potassium phosphate buffer, pH 6.6 and 0.05 M  $\text{MgSO}_4$ . Unless otherwise stated, 2,4-dinitrophenol was added at a final concentration of 0.4 mM. [ $^3\text{H}$ ]-Cyclic AMP (10.1 mCi/mmol) was diluted with cold cyclic AMP to yield [ $^3\text{H}$ ]-cyclic AMP having concentration between 20 and 160 mM and specific activities between  $6.6 \times 10^6$  cpm/mmol and  $0.25 \times 10^5$  cpm/mmol. These cyclic AMP solutions were used in 1/10 dilutions in the assay tubes. Incubation was carried out at 30°C for 4 min. The uptake was terminated by diluting the reaction mixture with 0.1 M LiCl. The vesicles were filtered through a membrane filter and washed with 0.1 M LiCl. The filter was dried, and placed in a vial containing 8 ml of scintillation cocktail (PPO, 5 mg; POPOP, 0.125 g; toluene, 1 L) and the radioactivity was measured in a Liquid Scintillation System LSS 20 (Electronic Corporation of India Limited).

Efflux of cyclic AMP was measured by following the uptake of [ $^3\text{H}$ ]-cyclic AMP by everted vesicles. Everted vesicles were prepared according to the method of Goldenbaum and Hall (1979). The normally oriented vesicles were diluted 10 fold in cold 0.05 M phosphate buffer, pH 7.0 and passed through French Pressure Cell (Aminco, USA) at 5000 lb/in $^2$ . The everted vesicles were collected in a chilled centrifuge tube and sedimented at 20000 *g* for 30 min. The pellet was resuspended in the same buffer to a volume equal to that which was originally taken.

The same criteria used by Goldenbaum and Hall (1979) was followed for detecting the conversion of vesicles of normal orientation by passage through French pressure cell to everted vesicles. The uptake of [ $^{14}\text{C}$ ]-L-leucine by each batch of everted vesicle preparation was measured and was found to be only 14–18% of that by normally

oriented vesicles. The fact that this value is similar to those reported for the uptake of [ $^{14}\text{C}$ ]-proline (Ninio and Tamamoto, 1974) and [ $^{14}\text{C}$ ]-serine (Goldenbaum and Hall, 1979) by everted vesicles suggests that our pressure-prepared vesicles were essentially everted.

## Results

### *Uptake of [ $^3\text{H}$ ]-cyclic AMP in membrane vesicles prepared from UV irradiated cells*

Cells were grown to late logarithmic phase in minimal medium containing casamino acids (0.025%) and glycerol (22 mM) in presence or absence of cyclic AMP (1.25 mM). Cells grown in presence of cyclic AMP and a part of cells grown without cyclic AMP were irradiated with UV light (540 ergs/mm<sup>2</sup>). Vesicles were prepared from the unirradiated and the two irradiated cell suspensions and uptake of [ $^3\text{H}$ ]-cyclic AMP for 4 min was measured in presence of various concentrations of cyclic AMP. It is clear from the results presented in figure 1 that UV irradiation of cells caused a substantial reduction in the uptake of [ $^3\text{H}$ ]-cyclic AMP in vesicles prepared from them as compared to that in vesicles from unirradiated cells. The presence of cyclic AMP in the medium during growth partially reduced the inhibitory effect of UV on the uptake of [ $^3\text{H}$ ]-cyclic AMP. The inhibitory effect of UV irradiation and its reduction by cyclic AMP were observed with all the concentrations of cyclic AMP present in the assay mixture.

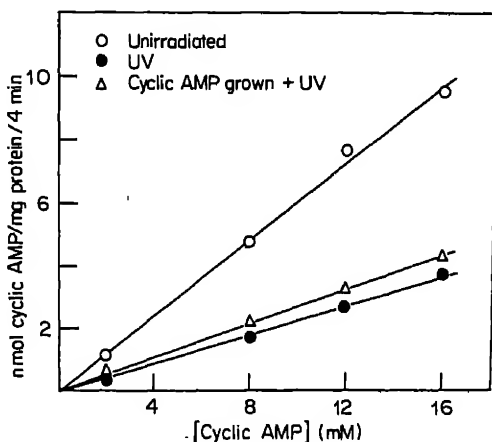


Figure 1. Effect of UV irradiation of *E. coli* B/r cells on the uptake of [ $^3\text{H}$ ]-cyclic AMP. Cells were grown in presence and absence of cyclic AMP (1.25 mM), irradiated with UV light (540 ergs/mm<sup>2</sup>), and uptake of [ $^3\text{H}$ ]-cyclic AMP was measured in vesicles prepared from them.

### *Uptake of cyclic AMP in vesicles prepared from cells grown under various conditions*

To find out whether the uptake of [ $^3\text{H}$ ]-cyclic AMP changes under conditions of catabolite repression by glucose and its reversal by cyclic AMP, effect of growth of the cells on glucose and/or cyclic AMP on the uptake in vesicles prepared from them was

studied. *E. coli* B/r cells were grown in minimal medium containing casamino acids and glycerol (22 mM) or glucose (20 mM), both in presence and absence of cyclic AMP (1.25 mM). Vesicles were prepared from each of the four cell suspensions and the uptake of [ $^3$ H]-cyclic AMP for 4 min was measured in presence of various concentrations of cyclic AMP. The results are shown in figure 2. Uptake of [ $^3$ H]-cyclic AMP in vesicles prepared from cells grown on glucose was found to be significantly less than that in vesicles prepared from cells grown on glycerol. Cyclic AMP present during growth of *E. coli* B/r on either glycerol or glucose as the carbon source caused an enhancement of the uptake.

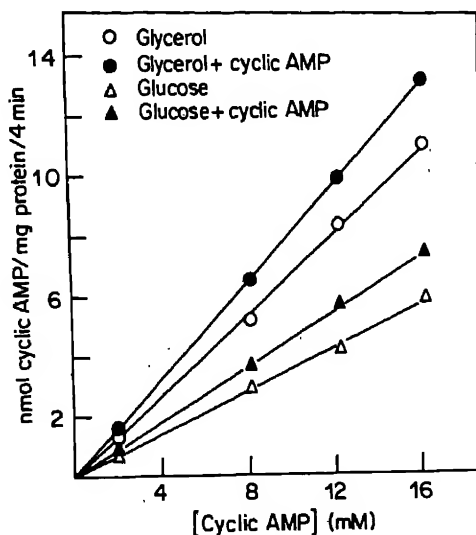


Figure 2. Effect of various growth conditions of *E. coli* B/r cells on the uptake of [ $^3$ H]-cyclic AMP.

Cells were grown on glycerol (22 mM) or glucose (20 mM), both in presence or absence of cyclic AMP (1.25 mM).

**Effect of UV irradiation on vesicles preloaded with [ $^3$ H]-cyclic AMP:** The vesicles were allowed to take up [ $^3$ H]-cyclic AMP for 20 min at 30°C in an uptake assay mixture, as already described under 'materials and methods' containing 8 mM [ $^3$ H]-cyclic AMP of a specific activity of  $1.65 \times 10^6$  cpm/ $\mu$ mol in the absence of 2,4-dinitrophenol. These vesicles loaded with [ $^3$ H]-cyclic AMP were centrifuged at 45,000 *g* for 30 min, washed and resuspended in 0.05 M phosphate buffer, pH 6.6, and irradiated with various doses of UV light. Unirradiated loaded vesicles were kept as control. Samples collected at various times after UV irradiation were filtered through millipore filters and washed with 0.1 M LiCl. Each filter was dried and the radioactivity was measured. The results presented in figure 3 show that UV irradiation led to an efflux of [ $^3$ H]-cyclic AMP from preloaded vesicles, the decrease being more with increasing dose of UV light.

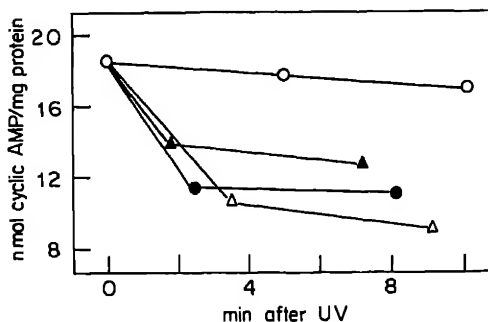


Figure 3. Efflux of [ $^3\text{H}$ ]-cyclic AMP from preloaded vesicles following UV irradiation.

Normal vesicles were preloaded with [ $^3\text{H}$ ]-cyclic AMP by allowing them to take it up for 20 min in the absence of 2,4-dinitrophenol. Samples were collected at various times as indicated after irradiating the vesicles with UV light of the following doses; 0 ergs/mm<sup>2</sup> (○), 180 ergs/mm<sup>2</sup> (△), 540 ergs/mm<sup>2</sup> (●), and 720 ergs/mm<sup>2</sup> (▲).

#### Efflux of [ $^3\text{H}$ ]-cyclic AMP using everted vesicles prepared from UV irradiated cells

To study the efflux of cyclic AMP under conditions of UV light induced catabolite repression, the uptake of cyclic AMP was measured in everted vesicles prepared from UV irradiated *E. coli* B/r cells. Cells grown on glycerol both in presence and absence of cyclic AMP were irradiated with UV (540 ergs/mm<sup>2</sup>) and vesicles were prepared from them. The vesicles were everted by passing the normally oriented vesicles through French Pressure Cell. The incubation mixture for the study on uptake of [ $^3\text{H}$ ]-cyclic AMP in everted vesicles was the same as that in normal vesicles except that 2,4-dinitrophenol was not present and ascorbate-PMS was used as an energy source. Varying amounts of cyclic AMP and [ $^3\text{H}$ ]-cyclic AMP were used in such a way that the specific activity in each assay tube remained constant at  $1.65 \times 10^6$  cpm/ $\mu\text{mol}$ . The uptake of [ $^3\text{H}$ ]-cyclic AMP in everted vesicles was taken as a measure of its efflux. The results presented in figure 4 clearly show that UV irradiation caused an inhibition of the efflux of [ $^3\text{H}$ ]-cyclic AMP, and the growth of the cells in presence of cyclic AMP prior to irradiation partially decreased this inhibitory effect.

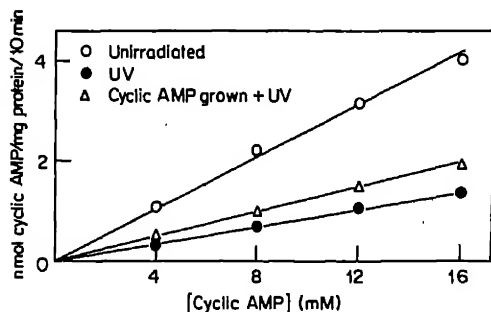


Figure 4. Effect of UV irradiation of *E. coli* B/r cells on the efflux of [ $^3\text{H}$ ]-cyclic AMP.

The experimental conditions were the same as described in legend to figure 1 except that the uptake was measured in vesicles everted by passing normally oriented vesicles through French Pressure Cell.

*Efflux of [ $^3\text{H}$ ]-cyclic AMP using everted vesicles prepared from the cells grown under various conditions*

Since the efflux of [ $^3\text{H}$ ]-cyclic AMP was found to be reduced under conditions of catabolite repression by UV, it was of interest to check whether catabolite repression by glucose caused any change in the capability of the everted vesicles to take up [ $^3\text{H}$ ]-cyclic AMP. Membrane vesicles were prepared from cells grown on glycerol or glucose, in presence and absence of cyclic AMP. Everted vesicles prepared from them were used to study the efflux. Everted vesicles prepared from cells grown on glucose had a significantly less capacity for the uptake of [ $^3\text{H}$ ]-cyclic AMP than those prepared from glycerol grown cells (figure 5). The efflux was increased by cyclic AMP not only in vesicles from cells under condition of catabolite repression by glucose but also in vesicles from glycerol grown cells.

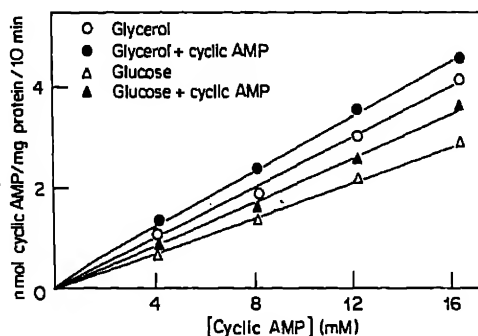


Figure 5. Effect of various growth conditions of *E. coli* B/r cells on the uptake of [ $^3\text{H}$ ]-cyclic AMP.

The experimental conditions were the same as described in legend to figure 2.

## Discussion

The aim of the present work was to study the transport of cyclic AMP across *E. coli* membrane under condition of UV light induced catabolite repression. The effect of UV irradiation of preloaded vesicles in causing efflux of [ $^3\text{H}$ ]-cyclic AMP (figure 3) supported the idea that intracellular cyclic AMP is excreted into the suspending medium following irradiation of the cells (Bhatnagar and Bhattacharya, 1982). However, since the membrane vesicles do not have the capacity to synthesise L-arabinose isomerase in response to the inducer (data not presented), it was not possible to detect catabolite repression induced by UV irradiation. The purpose to have the same condition of catabolite repression by UV in the vesicles was achieved by irradiating the cells prior to vesicle preparation.

Goldenbaum and Hall (1979) showed that the uptake of cyclic AMP is by facilitated diffusion whereas the efflux is by active transport. They postulated that both uptake and efflux of cyclic AMP are mediated by the same carrier molecules. The lower uptake and efflux of cyclic AMP in vesicles from cells grown on glucose than in those from glycerol grown cells (figures 2 and 5) may be due to less number of cyclic AMP carriers.



The observation that vesicles from cells grown in presence of cyclic AMP show a higher cyclic AMP uptake (figure 2) and efflux (figure 5) than vesicles from cells grown without cyclic AMP is in confirmation with the results reported by Goldenbaum and Hall (1979). The effect of UV irradiation of cells in lowering the uptake (figure 1) and efflux (figure 4) of cyclic AMP in vesicles was similar to those by glucose. These results suggest that the number of carrier molecules for cyclic AMP transport had reduced under conditions of catabolite repression not only by glucose but also by UV irradiation. However, the inhibitory effect of UV was significantly more than the lowering of the uptake and efflux in vesicles prepared from glucose grown cells as compared to those in vesicles from glycerol grown cells. Furthermore, the stimulatory effect of the presence of cyclic AMP during growth on the cyclic AMP transport was much less under conditions of catabolite repression by UV than under condition of catabolite repression by glucose. The reason for this may be that glucose exerts its effect by causing catabolite repression whereas UV irradiation may exert its effect in different ways other than catabolite repression.

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## Diverse effects of formate on the assimilatory metabolism of nitrate and nitrite in *Rhizobium*

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**Abstract.** Two strains of *Rhizobium*, cowpea *Rhizobium* 32H1 and *Rhizobium japonicum* CB 1809, showed a marked stimulation in growth on addition of formate to the minimal medium containing nitrate as the sole source of nitrogen. The amount of accumulated nitrite and specific nitrate reductase activity was much higher in cultures supplemented with formate than in the control medium. In contrast, growth, consumption of nitrite and specific nitrite reductase activity in minimal medium + nitrite was greatly reduced by the addition of formate. A chlorate resistant mutant (*Chl*-16) was isolated spontaneously which contained a nitrite reductase which was not inhibited by formate. The results suggest that formate serves as an electron donor for nitrate reductase and inhibits nitrite assimilation in *Rhizobium*.

**Keywords.** Formate; nitrate reductase; nitrite reductase; *Rhizobium*.

### Introduction

*Rhizobium* assimilates nitrate and nitrite by inducing nitrate and nitrite reductases when the cells are grown in media containing nitrate or nitrite as the sole source of nitrogen (Bergersen, 1961; Sik and Barabas, 1977). The reduction of nitrate and nitrite consumes a large amount of energy and electrons for the induction are derived from various physiological electron donors such as formate, lactate, NADH etc. (Orth *et al.*, 1980). Formate serves as a good electron donor for nitrate reduction in *Escherichia coli* (Cole and Wimpenny, 1968) and *Pseudomonas denitrificans* (Radcliffe and Nicholas, 1970). However, Nishimura *et al.* (1980) reported that formate inhibits nitrate reductase activity in *P. nitrificans*. The present communication describes the effect of formate on the assimilation of nitrate and nitrite by two strains of *Rhizobium* under culture conditions.

### Materials and methods

Cultures of *Rhizobium* (strains 32H1 and CB 1809) were obtained as a gift from Dr. A. H. Gibson, CSIRO, Canberra, Australia and maintained on yeast-extract-mannitol agar slants (Vincent, 1970). Bacteria were grown in minimal medium (Ludwig and Signer, 1977) supplemented with 10 mM nitrate or 0.1 mM nitrite. Sodium formate was added in required concentrations after Millipore (pore size 0.22 µm) filter

sterilization. The media with and without formate are designated as formate medium and control medium respectively.

Growth was monitored turbidimetrically at 420 nm in a spectrophotometer (Spectronic 20, Bausch and Lomb, USA). The amount of nitrite accumulated or consumed was determined by the diazo-coupling method of Nicholas and Nason (1957). Specific nitrate and nitrite reductase activities were determined according to Pagan *et al.* (1977) except that  $\text{KNO}_3$  was replaced by  $\text{NaNO}_2$  in the reaction mixture when estimating nitrite reductase activity.

Chlorate resistant mutant was isolated according to Singh *et al.* (1980).

The protein was measured by Lowry's procedure (1951).

## Results

Growth of CB 1809 and 32H1 after 6 days in minimal medium +  $\text{NO}_3^-$  medium containing different concentrations of formate is shown in figure 1. Cell density of CB 1809 was higher at all the concentrations of formate added to the minimal medium +  $\text{NO}_3^-$  medium over control. However, growth of 32H1 was stimulated only upto 4 mM formate and decreased at higher concentrations. The growth pattern of 32H1 and CB 1809 was almost the same to that of in the control medium in early logarithmic phase. Sudden proliferation of cells occur at the exponential phase. At this stage, the absorbance of the formate medium (4 mM formate) was almost twice that of the control medium.

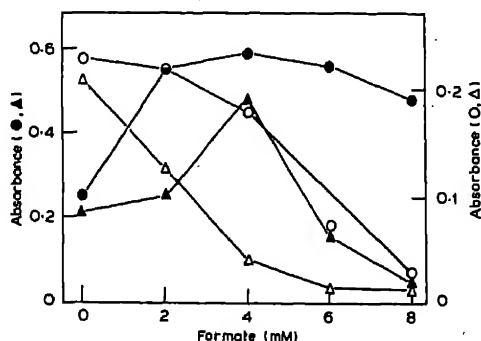


Figure 1. Effect of different concentrations of sodium formate on the growth of CB 1809 in minimal medium +  $\text{NO}_3^-$  (●); +  $\text{NO}_2^-$  (○); and growth of 32H1 in minimal medium +  $\text{NO}_3^-$  (▲); +  $\text{NO}_2^-$  (△).

Growth in nitrite medium was reversibly affected by addition of formate. The cell density of CB 1809 and 32H1 in minimal medium +  $\text{NO}_2^-$  medium decreases gradually by the addition of increasing concentrations of formate (figure 1). The inhibition by formate was much greater in 32H1 than in CB 1809.

The specific activity of nitrate reductase of 32H1 and CB 1809 was enhanced, and that of nitrite reductase was inhibited upon the addition of formate to the nitrate or nitrite medium (table 1).

**Table 1.** Effect of formate on the activities of nitrate reductase and nitrite reductase

Rhizobium strain	Specific activity <sup>a</sup>			
	Nitrate reductase		Nitrite reductase	
	Control	Formate	Control	Formate
CB 1809	806.2	1107	138.6	58.4
32H1	124.4	196.3	196.0	10.5

<sup>a</sup> n mol nitrite formed or consumed per hour per mg of protein.

Thirty two chlorate resistant mutants were isolated and tested for nitrate reductase activity. They failed to grow and show nitrate reductase activity in minimal medium +  $\text{NO}_3^-$  medium. However, their growth was unaffected in  $\text{NO}_2^-$  supplemented medium. When these mutants were inoculated in this  $\text{NO}_2^-$  medium containing 4 mM formate, growth of all the mutants except *Chl*-16 was inhibited by formate. The inhibition of growth of *Chl*-16 in nitrite medium containing formate was not observed. This strain (*Chl*-16) also showed comparable nitrite reductase activity in both minimal +  $\text{NO}_2^-$  or  $\text{NO}_2^-$  + formate (4 mM) media. The presence of formate dehydrogenase activity in *Chl*-16 was examined to explore the possibility. The formate was decomposed in the medium. The dehydrogenase could not be detected.

## Discussion

In the presence of nitrate, a membrane bound formate nitrate reductase system is induced in *Escherichia coli* (Glaser and DeMoss, 1971) which oxidizes formate by formate dehydrogenase and reduces nitrate to nitrite by nitrate reductase. Formate therefore serves as a physiological electron donor for nitrate reduction. However, the role of formate as an electron donor for nitrate reduction in *Pseudomonas denitrificans* is controversial because Radcliffe and Nicholas (1970) reported that formate promotes nitrate reduction by supplying electrons whereas according to Nishimura *et al.* (1980), nitrate reduction is depressed by formate. They also reported that nitrite consumption is enhanced by formate. The effect of formate on nitrite and nitrate reduction observed here are just the reverse of the results obtained by Nishimura *et al.* (1980). Since it is now generally accepted that nitrate reduction is considered to couple with electron requiring reactions, formate in the two strains of *Rhizobium* (CB 1809 and 32H1) serves as the electron donor for nitrate reduction. Formate is oxidised by formate dehydrogenase and the electrons released are accepted by nitrate to be reduced to nitrite by nitrate reductase.

The inhibition of nitrite reduction by formate seems to be an unexpected phenomenon. The concentrations of nitrite as well as formate are not toxic individually. Although formate is known to serve as an effective electron donor for nitrite reduction (Nishimura *et al.*, 1979), the assimilation of nitrite by 32H1 and CB 1809 is inhibited by even 2 nM formate. It appears that formate, or any product formed after its oxidation

is responsible for the observed inhibition. Mutant *Chl-16*, defective in nitrate reductase, showed effective nitrite assimilation in the presence of formate, even at 4 mM concentration, and fails to show formate dehydrogenase activity. Its inability to show inhibition of nitrite assimilation by formate, and defective formate dehydrogenase, clearly indicates that the product of formate oxidization, formed by formate dehydrogenase is responsible for the observed inhibition of nitrite reduction.

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## Ureide biogenesis and the enzymes of ammonia assimilation and ureide biosynthesis in nitrogen fixing pigeonpea (*Cajanus cajan*) nodules

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**Abstract.** Allantoic acid production from IMP, XMP, inosine, xanthosine, hypoxanthine, xanthine, uric acid and allantoin was investigated by incubating each of these substrates with *Cajanus cajan* cytosol and bacteroid fractions separately in the presence and absence of  $\text{NAD}^+$  and allopurinol. Allantoic acid synthesis by bacteroid fraction could only be observed with uric acid and allantoin as substrates. Addition of  $\text{NAD}^+$  or allopurinol to the reaction mixtures had no effect. However, with cytosol fraction, allantoic acid was produced by each of these substrates, with maximum rate with allantoin. With  $\text{NAD}^+$  or with allopurinol, allantoic acid was produced only with uric acid and allantoin as substrates. NADH production with cytosol fraction could again be observed with all the substrates. Except with uric acid and allantoin, allopurinol completely inhibited NADH formation. Regardless of the presence or absence of allopurinol, none of the substrates exhibited significant activity with bacteroid fraction. Based on the activities of glutamine synthetase, glutamate synthase, glutamate dehydrogenase, aspartate aminotransferase, asparagine synthetase, nucleotidase, nucleosidase, xanthine dehydrogenase, uricase and allantoinase and their intracellular localisation in various nodule fractions, a probable pathway for the biogenesis of ureides in pigeonpea nodules has been proposed.

**Keywords.** Pigeonpea; *Cajanus cajan*; nodules; ureide biogenesis; intracellular location.

### Introduction

In many of the tropical legumes including pigeonpea, most of the nitrogen fixed in nodules is translocated in the form of ureides, allantoin and allantoic acid (Herridge *et al.*, 1978; Pate *et al.*, 1978; Streeter, 1979; McClure and Israel, 1979; Luthra *et al.*, 1981; Sheoran *et al.*, 1982). These ureides are synthesised *via* a pathway involving biosynthesis of purines followed by oxidation and hydrolysis (Atkins *et al.*, 1980a, Schubert, 1981; Boland and Schubert, 1982). Studies on enzymes of purine biosynthesis in soybean nodules have demonstrated the presence of phosphoribosylpyrophosphate synthetase (EC 2.7.6.1), phosphoribosyl amidotransferase (EC 2.4.2.14), phosphoglycerate dehydrogenase (EC 1.1.1.95), serine hydroxymethyltransferase (EC 2.1.2.1) and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) (Schubert, 1981; Reynolds *et al.*, 1982a), indicating that purine biosynthesis in nodules parallels known pathways in animals and microorganisms. This assumption gained support when the specific activity of all these enzymes were found to increase markedly in soybean nodules during the onset of nitrogen fixation and ureide production (Reynolds *et al.*, 1982a). Moreover, their levels in soybean nodules were much greater than in nodules of lupin and pea, legumes, in which the main products of ammonium assimilation are amino

acid amides rather than ureides (Reynolds *et al.*, 1982b; Christensen and Jochimsen, 1983). Evidences also became available which indicated that purines synthesised *de novo* in the plant cells, were precursors for ureides in the nodules (Triplett *et al.*, 1980; Woo *et al.*, 1980, 1981; Atkins *et al.*, 1980b; Boland and Schubert, 1982). However, the mechanism for the assimilation of fixed N into ureides still remains to be clearly elucidated. In our recent studies (Luthra *et al.*, 1983a, b), we proposed a metabolic pathway for the assimilation of ureides into seed proteins in pigeonpea. In this communication, we propose a probable route for the formation of ureides from nucleotides in nodule cytosol fraction. Activities of various enzymes relative to acetylene reduction activity ( $N_2$  fixation) are also given in an attempt to demonstrate that these enzymes may be involved in amide synthesis and ureide biogenesis in nodules of pigeonpea plants.

## Materials and methods

### *Plant material*

The crop of pigeonpea (*Cajanus cajan* L.) cv. UPAS-120, was raised in sand culture in earthen pots under natural conditions of light and humidity as described earlier (Sheoran *et al.*, 1982). At weekly intervals, each pot was supplied with 250 ml of N-free nutrient solution (Wilson and Reisenaur, 1963). On other days, the pots were irrigated with tap water. Nodules from 40 to 45 day old plants were harvested and used immediately.

### *Preparation of cytosol, bacteroid and proplastid fractions*

Cytosol fraction from freshly harvested nodules was prepared essentially according to Herridge *et al.* (1978). Bacteroids were isolated according to the method of Triplett *et al.* (1980) and proplastids were separated by following the procedure of Boland and Schubert (1983). Freshly harvested nodules (3 g) were broken in 10 ml 0.1 M Tricine buffer (pH 8.0), containing 0.4 M sucrose, 10 mM KCl, 10 mM EDTA, 5 mM dithiothreitol, 1 mM  $MgCl_2$  and 2 mM reduced glutathione, and filtered through cheese cloth. The brei obtained was centrifuged at 1650 *g* for 4 min. The darker red fluid obtained above the bacteroids was removed carefully and layered on a step gradient comprising a 5 ml band of 0.1 M Tricine-KOH (pH 8.0) containing 0.8 M sucrose, 2 mM DTT, 2 mM reduced glutathione, and 10 mM KCl, and a second 10 ml band of 2 M sucrose in 0.1 M Tricine-KOH (pH 8.0). The step gradient was centrifuged at 9000 *g* for 30 min in a swinging bucket rotor (Beckman Ultracentrifuge). The pellicle at the interface between the two sucrose layers was withdrawn and referred to as the proplastid fraction. The purity of the three fractions was monitored by assaying the marker enzymes.

### *Enzyme assays*

Nitrogenase (EC 1.18.2.1) activity in freshly harvested nodulated roots was determined



as described earlier (Luthra *et al.*, 1983b). Other enzymes were assayed separately in each fraction. Glutamine synthetase (EC 6.3.1.2), glutamate dehydrogenase (EC 1.4.1.4), uricase (EC 1.7.3.3) and allantoinase (EC 3.5.2.5) were assayed as described previously (Sheoran *et al.*, 1981). Glutamate synthase (EC 1.4.1.14) was assayed as described by Boland *et al.* (1978). Asparagine synthetase (EC 6.3.1.1) and aspartate aminotransferase (EC 2.6.1.1) were assayed by following the procedures of Rogenes (1975) and Bergmeyer and Bernt (1973), respectively. Xanthine dehydrogenase (EC 1.2.1.37) was measured by the rate of reduction of  $\text{NAD}^+$  in the presence of xanthine (Triplett *et al.*, 1982). Nucleotidase (EC 3.1.3.5) was assayed by measuring the release of inorganic phosphate from XMP. The assay mixture in a final volume of 1.5 ml contained: 100 mM Tris-maleate buffer (pH 5.3), 12 mM  $\text{MgCl}_2$ , 3 mM XMP, 1 mg bovine serum albumin and enzyme preparation. The mixture was incubated at 37°C for 15 min and the reaction terminated by adding 1 ml of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation and the inorganic phosphate estimated in the supernatant by the method of Ozbun *et al.* (1973). Nucleosidase (EC 3.2.2.1) was assayed by coupling the activity of XDH present in the enzyme preparation and following NADH production spectrophotometrically at 340 nm (Triplett *et al.*, 1982).

The marker enzymes phosphoenolpyruvate carboxylase (EC 4.1.1.31) for cytosol fraction,  $\beta$ -hydroxybutyrate dehydrogenase (EC 1.1.1.30) for bacteroid fraction and phosphoglycerate dehydrogenase for proplastid fraction were assayed according to the procedures of Hatch (1972), Wong and Evans (1971) and Boland and Schubert (1983), respectively.

Allantoic acid production from the different substrates used was measured as described earlier (Luthra *et al.*, 1983b). Protein after trichloroacetic acid precipitation was determined according to Lowry *et al.* (1951).

## Results and discussion

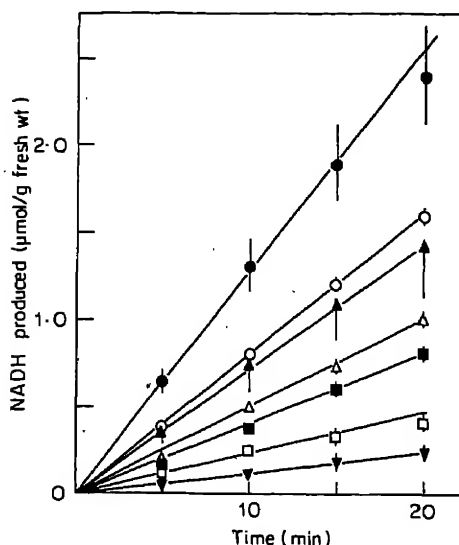
Ureide synthesis was assayed in bacteroid and cytosol fractions of pigeonpea nodules using several suspected precursors of allantoic acid synthesis. Allantoic acid could be produced from IMP, XMP, inosine, xanthosine, hypoxanthine, xanthine, uric acid and allantoin by cytosol fraction in the presence of  $\text{NAD}^+$  (table 1). The maximum production was observed with allantoin followed by uric acid, xanthosine, inosine, hypoxanthine, xanthine, XMP and IMP. Without  $\text{NAD}^+$  or with allopurinol, allantoic acid was produced only with uric acid or allantoin as substrate. The rate was again higher with allantoin than with uric acid. Allantoic acid synthesis by bacteroid fraction could only be observed with uric acid and allantoin as substrates. Addition of  $\text{NAD}^+$  or allopurinol to the reaction mixtures had no effect. With this fraction also, more allantoic acid was produced when allantoin was used as the substrate than with uric acid.

Using the same substrates, NADH production was followed spectrophotometrically at 340 nm. With cytosol fraction, rate of NADH production was linear for upto 20 min (figure 1) and maximum production was observed when hypoxanthine was used as the substrate. Low rates were obtained with IMP, XMP, uric acid or allantoin as substrates

**Table 1.** Allantoic acid production by cytosol and bacteroid fractions of pigeonpea nodules.

Substrate	p mol/mg protein/min					
	-NAD <sup>+</sup>	Cytosol +NAD <sup>+</sup>	+Allopurinol	-NAD <sup>+</sup>	Bacteroid +NAD <sup>+</sup>	+Allopurinol
IMP	0	108	0	0	0	0
XMP	0	125	0	0	0	0
Inosine	0	154	0	0	0	0
Xanthosine	0	178	0	0	0	0
Hypoxanthine	0	136	0	0	0	0
Xanthine	0	133	0	0	0	0
Uric acid	266	277	269	63	64	73
Allantoin	426	434	305	161	177	141

Reaction mixtures contained 0.2 mM substrate, 2.5 mM NAD<sup>+</sup> and the cytosol or bacteroid fraction containing about 1 mg protein. Allopurinol (0.2 mM) was added only to those reaction mixtures which contained NAD<sup>+</sup>. The mixtures were incubated at 30°C for upto 3 h and allantoic acid formed was estimated.



**Figure 1.** Time course of NADH formed by nodule cytosol fraction from various intermediates of ureide biosynthetic pathway. All assays included 2.5 mM NAD<sup>+</sup>, 0.2 mM substrate, 0.1 M potassium phosphate buffer (pH 7.8) and cytosol fraction containing about 1 mg protein.

(●), Hypoxanthine; (○) xanthine; (Δ), inosine; (▲) IMP; (□), XMP; (■), uric acid; (▼), allantoin.

(table 2). For all substrates except uric acid and allantoin, allopurinol completely inhibited NADH formation. Very little NADH was produced when uric acid and allantoin were used as substrates with cytosol fraction, and allopurinol had no effect on this synthesis. Regardless of the presence or absence of allopurinol, none of the substrates exhibited significant activity with the bacteroid fraction.

Results included in table 1 indicate that no substrate except uric acid and allantoin was utilised by bacteroid fraction for the formation of allantoic acid. Presence of NAD<sup>+</sup> or allopurinol also had no effect indicating that bacteroid is not the site for ureide synthesis. These results are contrary to those reported earlier by Tajima and

**Table 2.** NADH production by cytosol and bacteroid fractions of pigeonpea nodules from various intermediates of ureide biosynthetic pathway

Substrate	nmol NADH produced mg <sup>-1</sup> protein min <sup>-1</sup>			
	Cytosol		Bacteroid	
	- Allopurinol	+ Allopurinol	- Allopurinol	+ Allopurinol
IMP	2.5	0.2	0.0	0.0
XMP	1.4	0.0	0.0	0.0
Inosine	3.2	0.0	0.2	0.1
Xanthosine	5.0	0.2	0.6	0.2
Hypoxanthine	7.9	0.2	0.5	0.2
Xanthine	5.0	0.2	0.4	0.3
Uric acid	1.1	1.0	0.0	0.0
Allantoin	0.7	0.6	0.4	0.4

Reaction mixtures contained 0.2 mM substrate, 2.5 mM NAD and the cytosol or bacteroid fraction containing about 1 mg protein. NADH production was followed spectrophotometrically at 340 nm for 5 min at 25°C.

Yamamoto (1975), Tajima *et al.* (1977) and Fujihara and Yamaguchi (1978), where they proposed bacteroid as the probable site of ureide synthesis with xanthine oxidase as the xanthine oxidising enzyme. However, no xanthine oxidising activity could be detected in the present case in the bacteroid fraction and this prevented allantoinic acid production by all substrates except uric acid and allantoin. This was further confirmed when no NADH production was observed in bacteroid fraction in the presence of any of the substrates. Recent studies by Hanks *et al.* (1981) have indicated that uricase and allantoinase are associated with peroxisomes and microsomal fraction of soybean nodules, respectively. The presence of uricase and allantoinase in the bacteroid fraction observed here might have thus arisen due to the contamination of bacteroid fraction by these fractions in the preparation of bacteroids. Triplett *et al.* (1980) also observed some of the activity of uricase and allantoinase to be present in bacteroid fraction mainly due to contamination.

The results included in tables 1 and 2 further indicate that ureides arise mainly in cytosol fraction. Since in the absence of NAD<sup>+</sup> or presence of allopurinol, none of the substrates except uric acid and allantoin could produce allantoinic acid or NADH, cytosol ureide synthesis from the products of *de novo* purine biosynthesis require xanthine dehydrogenase activity. This was confirmed by the observation that the presence of allopurinol or absence of NAD<sup>+</sup> completely blocked the pathway. However, allantoinic acid production from uric acid and allantoin was unaffected by NAD<sup>+</sup> or allopurinol, indicating that oxidation of these substrates followed xanthine oxidation.

To further supplement these results, various enzymes involved in ammonium assimilation and ureide biosynthesis were assayed in nodule cytosol fraction and their activities expressed relative to the activity of nitrogenase (figure 2). The results clearly indicate that all the enzymes required for the initial assimilation of ammonium into glutamine and other amino acids, as well as for the biosynthesis of ureides from the products of purine synthesis were present in quite high amounts in cytosol fraction.

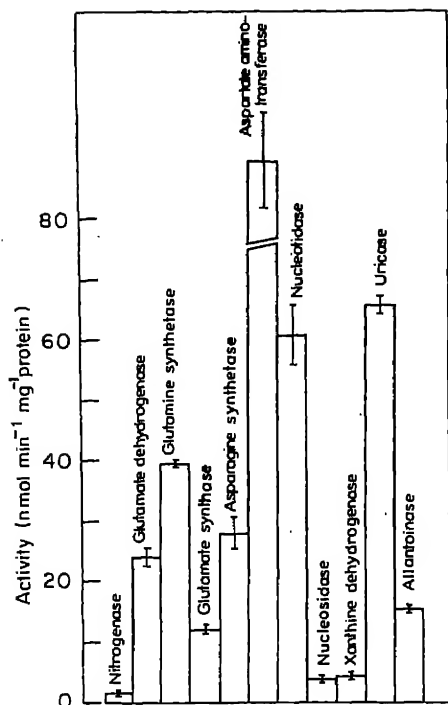
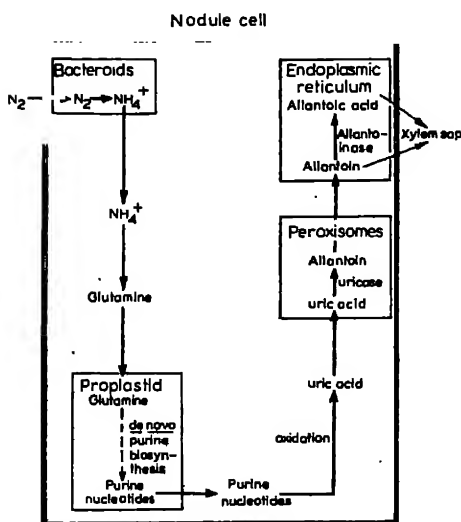


Figure 2. Enzyme activities in cytosol fraction of pigeonpea nodules relative to nitrogenase activity.

Based on all these observations, a probable pathway for biosynthesis of ureides in pigeonpea nodules could be visualised as follows: Atmospheric nitrogen is reduced to the level of ammonium by bacteroid nitrogenase. This ammonium is then transported across the bacteroidal membrane and is converted to glutamine in the cytosol by glutamine synthetase. Glutamate synthase, aspartate amino transferase and asparagine synthetase may be involved in the synthesis of amino acids and amides particularly glutamate, aspartate and asparagine etc. Glutamine and aspartate etc are then utilised for the biosynthesis of purines by well known reactions. The end product of purine biosynthesis, IMP, may be converted to XMP, by IMP dehydrogenase. This enzyme has recently been detected in soybean nodule cytosol by Shelp and Atkins (1983). IMP or XMP may be dephosphorylated by a nucleotidase to inosine or xanthosine, respectively. These could then be converted to hypoxanthine and xanthine by the enzyme nucleosidase. We could detect the activity of nucleotidase and nucleosidase in the cytosol fraction, but the activity of nucleosidase was comparatively much less than that of nucleotidase. Hypoxanthine and xanthine could then be oxidised to uric acid by xanthine dehydrogenase. Uric acid and allantoin in turn are oxidised by uricase and allantoinase, respectively to the end product, allantoinic acid. All these enzymes were detected in the nodule cytosol.

To obtain information about the probable intracellular localisation of these enzymes, we isolated bacteroidal, proplastid and cytosol fractions from pigeonpea nodules by differential centrifugation and sucrose density gradient method. The purity of these fractions was examined by marker enzymes *i.e.* phosphoglycerate de-



**Figure 3.** Proposed ureide biosynthetic reactions and their probable site of localisation in pigeonpea nodules.

hydrogenase for proplastid;  $\beta$ -hydroxybutyrate dehydrogenase for bacteroid; and phosphoenolpyruvate carboxylase for cytosol. The results of this experiment gave interesting information in that glutamine synthetase was localised mainly in cytosol and was absent from proplastid and bacteroidal fractions. Similarly, the enzymes of ureide biosynthesis from purines i.e. nucleotidase, nucleosidase, XDH, uricase and allantoinase were also present mainly in cytosol fraction. Hanks *et al.* (1981) while investigating the intracellular localisation of enzymes of ureide biosynthesis in soybean nodules, confirmed the presence of uricase in peroxisomes and that of allantoinase in microsomal fraction. Similarly, in a recent study, Boland *et al.* (1982) indicated the presence of enzymes of purine biosynthesis mainly in proplastids indicating that the complete pathway for purine biosynthesis is located in proplastids. This was confirmed when purine biosynthesis *in vitro* by a proplastid fraction from soybean (Boland and Schubert, 1983) and Cowpea (Shelp *et al.*, 1983) nodules was demonstrated. Putting all this information together, a complete pathway of ureide biogenesis from the products of nitrogen fixation in pigeonpea nodules could be visualised as depicted in figure 3. According to this scheme, ammonium is assimilated to the level of glutamine in cytosol. This along with aspartate and other intermediates are utilised for the biosynthesis of purines in proplastids. The end products of purine biosynthesis are oxidised to the level of uric acid in cytosol. Two of the last steps may be occurring in peroxisomes and endoplasmic reticulum, respectively.

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## Adrenocorticotrophin secreting cells in the hypophysis of the brown spiny mouse *Mus platythrix* (Bennett)

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**Abstract.** Adrenocorticotrophin secreting cells are identified in the hypophysis of the brown spiny mouse *Mus platythrix* by conventional methods of light microscopy. Quantitative data showed that certain smaller acidophilic cells in the *pars distalis*, under conditions provoking their hypersecretion such as unilateral adrenalectomy and metopirone treatment, increase in number and size from the pre-existing corticotrophs. There is no evidence for the transmigration of these cells from the chromophobes, basophils or any other cell type. The *pars intermedia* revealed two types of cells of which the type II cells are histochemically identical to adrenocorticotrophin secreting cells of the *pars distalis*.

**Keywords.** mouse; adrenocorticotrophic secreting cells; metopirone; unilateral adrenalectomy; cold stress; histochemistry.

### Introduction

According to Baker and Gross (1978), the corticotrophs of the mouse have never been studied from the tissue sections prepared for differential chemical staining. In other species of rodents as well, the corticotrophs are the least understood of all the cell types of the pituitary. The tinctorial affinities of these cells are ill-defined (Totsuka, 1967). Many investigators considered them acidophilic (Herlant and Massant, 1976), basophilic (Baker *et al.*, 1972) and chromophobic (Nakane, 1970). Electron microscopic observations added another dimension to this controversy. Acidophils (Girod, 1964), amphiphils (Murphy and James, 1976), adrenocorticotrophin (ACTH), follicle stimulating hormone (FSH) cells (Moriarty and Garner, 1977) and thyrotrophs (Kurosomi, 1968) are implicated in ACTH secretion in several electron microscopic studies. The purpose of the present investigation is to describe the morphology, tinctorial affinities, qualitative and quantitative changes in ACTH secreting cells under normal and altered physiological states of adrenal gland in *Mus platythrix*.

### Materials and methods

Adult *Mus platythrix* of both sexes were collected from Kadkola, 8 km from Mysore

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Abbreviations used: ACTH, Adrenocorticotrophin; PAS, Periodic acid-Schiff; OG, Orange-G; PbH, lead haematoxylin; AF, aldehyde fuchsin; FSH, follicle stimulating hormone; MSH, melanocyte stimulating hormone; STH, somatotrophic hormone.

and acclimatised to the laboratory conditions for a period of 3–4 days. They were kept in groups of 4–5 individuals per cage (14' × 12' and 12'), and were maintained on grain and meat with free access to drinking water. These mice were divided into the following experimental groups.

**Group I:** A batch of four mice was subjected to cold stress at 4°C for 24 h and were sacrificed thereafter.

**Group II:** A batch of 16 mice was unilaterally adrenalectomised under light ether anesthesia (Zarrow *et al.*, 1964) and sacrificed on day 7, 14, 28 and 56 post operatively with 4 animals in each interval. Four sham operated animals served as controls.

**Group III:** Three batches of mice, each consisting of three individuals were given subcutaneous injections of metopirone (Ciba, England), 5 mg/day for 7, 14 and 28 days. A group of four distilled water-injected mice served as controls. All the mice were sacrificed 24 h after the last injection.

The protocol followed to avoid stress in the mice was similar to that followed by Madhyastha and Dutt (1978). Further, care to avoid stress-induced changes in ACTH secretion was taken by sacrificing the mice by decapitation within a few seconds after careful handling in each experiment.

Pituitaries and adrenals of all experimental groups were fixed in Hollande-Bouin-sublimate, formal-sublimate, Bouin's fluid and chilled 80% alcohol. Standard staining techniques were followed to identify ACTH secreting cells (table 1). Three to 5  $\mu$ m thick sections of adrenals Bouin's fluid fixed of experimental and control mice were stained by Periodic acid-Schiff (PAS)/haematoxylin method. Values of mean width of the cortex and medulla of adrenals were recorded from 10 readings of histological sections using an ocular micrometer at 50X. The cortex/medulla ratio was calculated.

**Table 1.** Tinctorial affinities of ACTH secreting cells and type I and type II cells of the *pars intermedia* of *Mus platythrix*.

Fixatives	Staining techniques	Reference	Tinctorial affinities		
			ACTH Cells	Type I cells	Type II cells
Hollande-Bouin	PAS/OG	McManus, 1946	Pale orange	Weakly PAS +ve	Weakly PAS -ve
-do-	Trichrome	Cleveland and Wolfe, 1932	Erythro-sinophilic	Pale blue	Moderately blue
-do-	Tetrachrome	Herlant, 1960	-do-	Blue	Blue
Formol-sublimate	PAS/AB/PAS/OG	Heath, 1965	Pale orange	Blue	Blue
-do-	PbH	MacConnail, 1947	PbH +ve	PbH +ve	PbH +ve



Three to 5  $\mu$ m paraffin sections of pituitaries were subjected to histochemical analysis as given in table 2.

**Table 2.** Histochemical analysis of ACTH secreting cells and *pars intermedia* cells of *Mus platythrix*.

Fixative	Histochemical test	Reference	Reaction in		
			ACTH cells of PD	Type I cells of PI	Type II cells of PI
Bouin	PAS	McManus, (1946)	—	+	+
-do-	Performic acid/alcian blue	Pearse, (1972)	++	+	++
Carnoy	HgBPB	Pearse, (1972)	+	+	+
-do-	Trypsin digestion/HgBPB	Pearse, (1972)	—	—	—
10% Neutral formalin	Fast green FCF	Alfert and Geschwind, (1953)	+++	+	+++
Carnoy	Methyl green/pyronin Y	Brachet, (1953)	++	—	++
-do-	Perchloric acid treatment and stained as above	Pearse, (1972)	—	—	—
-do-	Methyl green toluidine blue	Korson, (1951)	+	—	+
80% Chilled alcohol	Gomori's Alkaline phosphatase	Gomori, (1950)	+	—	+
-do-	Millon's reaction	Pearse, (1972)	++	++	++

## Results

### *Histology of normal pituitary*

The ACTH secreting cells in *M. platythrix* belong to serous category as described earlier by Madhyastha and Dutt (1978) and are found evenly distributed in the *pars distalis* except in 'sex zone'. The tinctorial affinities of them have been summarised in table 1. The shape of ACTH cells is varied, generally ovoid or rounded, but those which are in association with somatotrophs are cupped and stellate and their cytoplasm is clear and homogeneous. With PAS/Orange-G (OG) and Lead Haematoxylin staining techniques, the reaction in the cytoplasm is perceptible (figure 1). Occasionally erythrosinophilic granules are also seen in the cytoplasm. The Golgi region is inconspicuous in them. The nuclei of those cells are large and rounded.

The *pars intermedia* of the normal mouse consists of two varieties of closely packed cells. The first variety of these cells is abundant and uniformly distributed. They are large and elongated with vesicular nuclei and are stained weakly with PAS, aniline blue and aldehyde fuchsin. The cells of the second variety are relatively smaller than those of the first category and are often stellate in shape with a hyperchromatic nucleus (figure 2). They are generally found in groups, in the anterior and dorsal side of the *pars intermedia*. They reveal marked affinity with aniline blue, aldehyde fuchsin (AF) and lead haematoxylin (PbH).

### Histochemistry

Histochemically, the ACTH cells of the *pars distalis* and the type II cells of the *pars intermedia* are acidophilic in nature. Both of them are seen distinctly stained with fast green FCF, (figure 3) and alcian blue indicating the presence of basic proteins and cysteine respectively. With methyl green pyronin Y technique, pyronin stains the cytoplasm of ACTH cells and type II cells of *pars intermedia* but not that of type I cells. Both these cell types are also rich in alkaline phosphatase. The tests for arginine and tyrosine, however, gave positive reactions in the cytoplasm of both the cell types of *pars intermedia* and the ACTH cells as well.

### The adrenal gland

The adrenal gland of the control mouse consists of a capsule, the cortex and the medulla as in other mammals (figure 4). The outermost layer of the cortex, namely, the zona glomerulosa is made up of loops and balls of cells, the cytoplasm of which is markedly dense. The middle layer, namely, the zona fasciculata has a long column of cells, the cytoplasm of them is vacuolated and less dense. The nuclei of these cells are large. Mitotic figures are commonly seen in this region. In the innermost zona reticulata, the cells are found anastomosed and irregular in their distributional pattern. The cells are small, with cytoplasm and nuclei appearing densely stained. Vascularisation is rich in this region of the cortex.

### Cold stress

Hypertrophy was seen in some serous cells of mice maintained at 4°C for 24 h. They are voluminous and are found in groups, some of them occasionally possess cytoplasmic processes between the neighbouring cells, particularly the somatotrophs. The hypertrophied ACTH cells are highly conspicuous (figure 5).

### Unilateral adrenalectomy

One week after unilateral adrenalectomy, many significant changes are observed in the serous cells which are comparable to those seen in the cold stressed animals. They are voluminous and often are seen aggregated in groups (figure 6). Mitotic figures are seen occasionally. A moderate increase in the number of cells is noticed. The secretory granules of these cells are seen concentrated at the cell periphery. The nuclei are large containing one or two conspicuous nucleoli. Furthermore these cells increase in numbers, two weeks after operation. Mitotic stages among them appear to be

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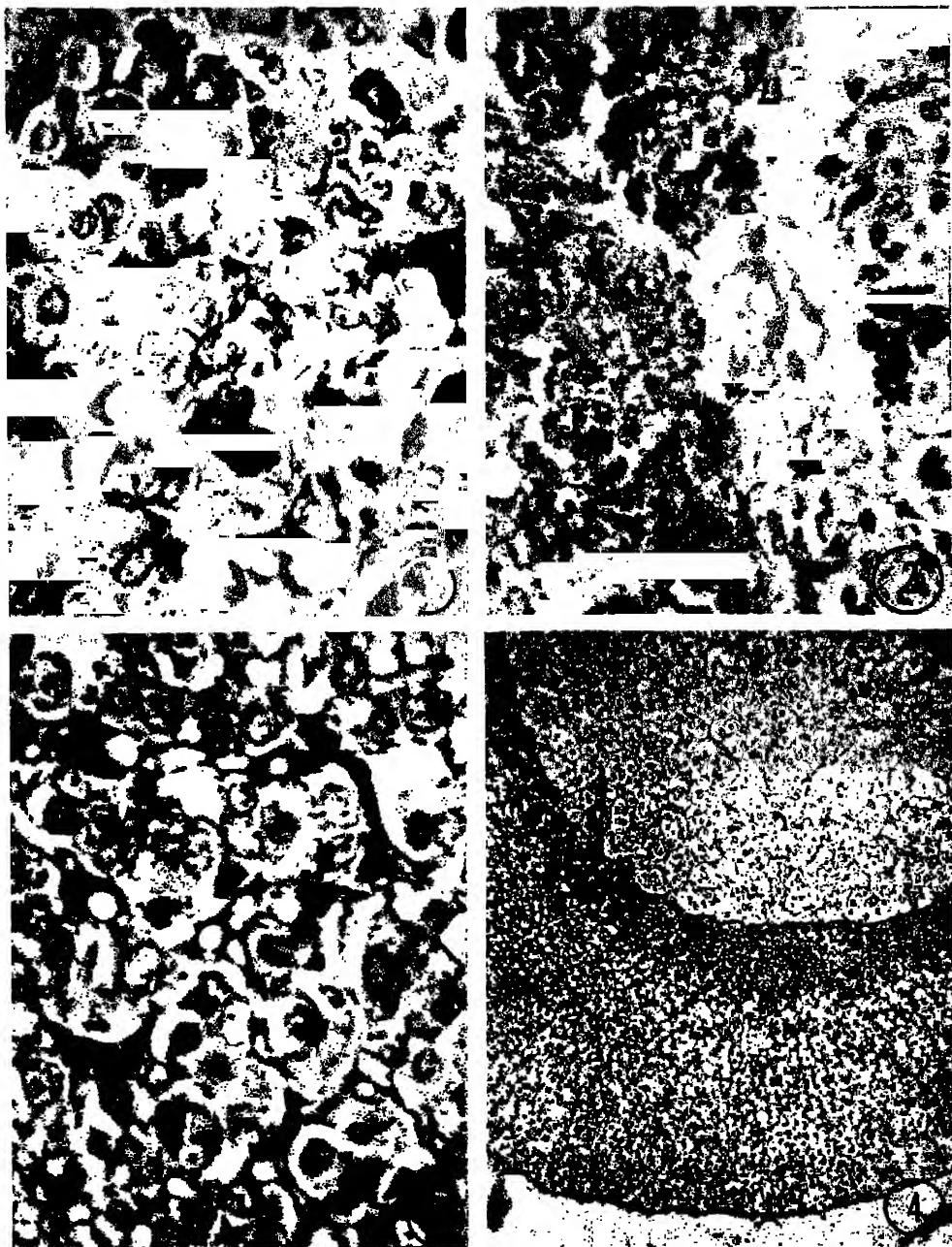
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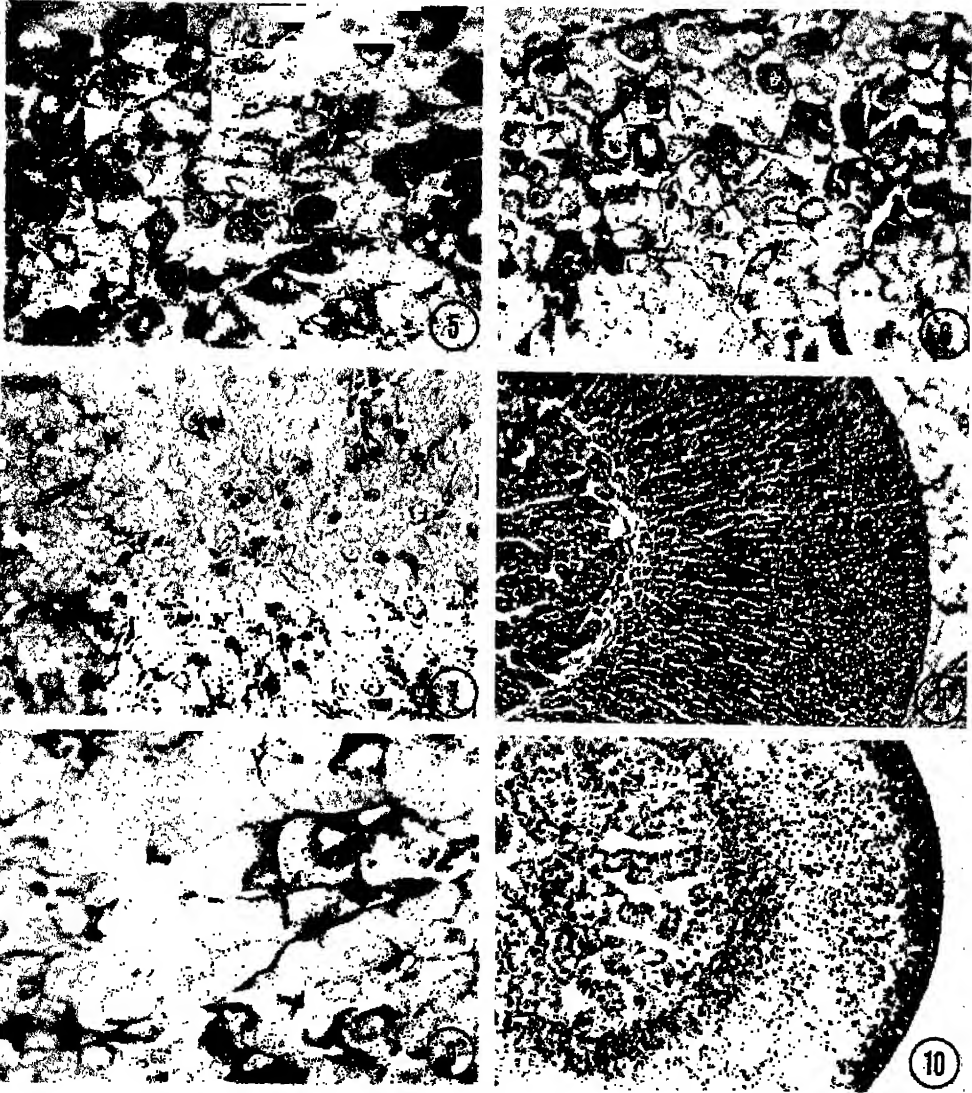
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Figures 1-4. 1. ACTH cells in the *pars distalis* of the normal male *M. platythrix*. X 600. 2. Type II cells in the *pars intermedia* of *M. platythrix*. X 600. 3. *Pars distalis* of *M. platythrix* showing fast green positive ACTH cells. X 600. 4. A portion of an adrenal gland of control mouse showing cortex and medulla. X 120.

prominent (figure 7). By four weeks after the operation, the cells are seen voluminous, their percentage reached a peak, which however declined after eight weeks.

A definite histological change is observed in the contralateral adrenal one week after unilateral adrenalectomy. The size of the cortex, particularly that of zona fasciculata



Figures 5-10. 5. A portion of the *pars distalis* of *M. platythrix* showing hypertrophied ACTH and TSH cells after cold-stress. X 600. 6. A group of ACTH cells in the *pars distalis* one week after adrenalectomy. X 800. 7. A portion of *pars distalis* showing ACTH cells in various stages of mitosis. X 400. 8. Adrenal cortex of contralateral adrenal gland increased in thickness 56 days after unilateral adrenalectomy. X 120. 9. Hypertrophied ACTH cells of the *pars distalis* after metopirone administration. X 800. 10. Degenerative adrenal cortex of the metopirone-treated mouse. X 120.

and the external part of the zona fasciculata are increased. The capsule of the adrenal is thickened one week after the operation. Increase in cortical cells appears to be a continuous process and by 56 days after unilateral adrenalectomy the contralateral adrenal cortex is almost doubled in thickness as compared to that of the control mouse (figure 8).

### Metopirone treatment

Metopirone induces hyperplasia and hypertrophy of those serous cells which showed response to unilateral adrenalectomy. The number of serous cells increases gradually from one week to four weeks of treatment with this drug. The mitotic figures among the serous cells are less frequent during first and second week after the treatment, later however they become increasingly common. By four weeks, a few serous cells become hyalinised, while others have revealed one or two vacuoles in their cytoplasm (figure 9) and these cells reached their peak of abundance (figure 11).

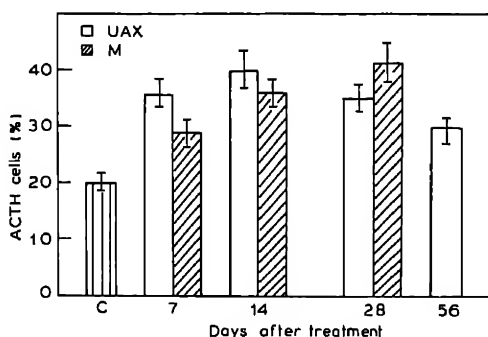


Figure 11. Histogram showing percentage of ACTH secreting cells in the control, unilaterally adrenalectomized and metopirone treated mice.

In striking contrast to that after unilateral adrenalectomy, metopirone treatment has resulted in destructive changes in the adrenal cortex. By one week after the treatment, the zona fasciculata and the zona reticulata showed histological signs of degeneration such as reduction in their size, loss in stainability of the cytoplasm and appearance of pycnotic nuclei. The thickness of the cortex, however, is not altered (table 3). By two

Table 3. Adrenal cortex/medulla (C/M) ratio in the unilateral adrenalectomized metopirone treated *Mus platythrix*.

Experiment	Control	C/M ratio			
		7 days	14 days	28 days	56 days
Unilateral adrenalectomy	1.1 ± 0.1	1.0 ± 0.2	1.3 ± 0.3	1.8 ± 0.2	2.3 ± 0.4
Metopirone treatment	1.1 ± 0.1	1.3 ± 0.5	0.82 ± 0.1	0.43 ± 0.05	—

± SEM; — the values too low to measure.

weeks after treatment the adrenal capsule is narrowed down and the glomerulosa layer becomes thickened. Many signet ring cells are visible among the cells of zona fasciculata and the zona reticulata. By four weeks the adrenal cortex undergoes a complete degeneration (figure 10). The cells appear indistinct and their nuclei are markedly pycnotic. Vacuoles are noticed in the cells of zona fasciculata layer.

## Discussion

Numerous cytophysiological studies have been made to identify the corticotrophs in rat (Bowie *et al.*, 1973). Yet the controversy, whether the anterior pituitary corticotroph is an acidophil, or a basophil or a chromophobe does not appear to be resolved completely (Yoshimura *et al.*, 1974). This discrepancy is partly due to their weak affinity to dyes and partly to certain species differences. For example, PbH seems to be a selective stain for ACTH cells in fish (Ball and Oliverau, 1966), in slender loris (Anandakumar, 1966), but in others such as human (Ryan *et al.*, 1977), musk shrew (Naik and Dominic, 1974) and mouse (Madhyastha and Dutt, 1979) it is known to stain thyrotrophs and gonadotrophs. Besides, the tinctorial affinities of ACTH cells in rat vary with the acid dye which is used and probably with the physiological status of the cells (Moriarty, 1973). In the present study, a category of serous cell type is seen weakly PAS positive and reacts intensely with alcian blue. These cells also stain markedly with PbH and show marked secretory activity and progressive degranulation in response to cold stress. In mammals cold stress is also known to influence thyrotrophs and STH cells, but in the wild mouse the presumptive corticotrophs can be easily distinguished from them by their small size and even distribution in the hypophysis. Further Madhyastha and Dutt (1978, 1981) have demonstrated earlier that the larger acidophils belong to either Luteotrophic hormone or Somatotrophic hormone category and the thyrotrophs to the mucoid variety.

Compensatory hypertrophy of the contralateral adrenal after unilateral adrenalectomy has been reported for the laboratory mouse (Belka-Grybek, 1963). Brokaw *et al.* (1950) showed that the compensatory hypertrophy of adrenal in rat paralleled changes in the pituitary acidophils. Subsequently, Girod (1964) and Bugnon *et al.* (1965) have shown that the partial adrenal insufficiency in rat causes hypersecretion of ACTH from the anterior pituitary. A similar response both in the pituitary and adrenal cortex is revealed in the present study. In *M. platytrix* the removal of left adrenal evoked a massive response in the hypophysis. A variety of serous cells which are weakly OG positive and PbH positive increased numerically upto four weeks after the operation. They also showed intense secretory activity in the cytoplasm. These cellular changes paralleled the compensatory hypertrophy of the right adrenal.

Several investigators have shown that the ACTH cells exhibit definite changes after metopirone treatment (Kurosomi and Kobayashi, 1966; Cameron and Poster, 1972 and Naik and Dominic, 1978). Metopirone, an inhibitor of  $17\beta$ -hydroxylase of adrenal cortex is known to block the synthesis of corticosteroid (Gaunt, 1965) which in turn brings about hypersecretion of ACTH in fish (Ball and Oliverau, 1966), in bat (Herlant and Klastersky, 1962), in rat (Bugnon *et al.*, 1965), in hamster (Girod and Dubois, 1976) and in musk shrew (Naik and Dominic, 1978). In *M. platytrix*, the metopirone results in gradual increase in the number and volume of ACTH cells. They are identical with the

serous variety which is influenced by the cold stress and unilateral adrenalectomy. Hence it is reasonable to assume that the serous cells influenced by the cold stress, unilateral adrenalectomy and metopirone treatment are the ACTH cells.

The corticotrophic activity has been reported in *pars nervosa* and *pars intermedia* as well (Dubois, 1972; Pantic and Simic, 1974 and Zimmerman and Kraicer, 1978). Despite the use of contemporary staining techniques there are difficulties in distinguishing ACTH cells from the melanocyte stimulating hormone (MSH) cells (Pantic and Simic, 1977). Dubois (1972) recognised immunohistochemically the corticotrophs and MSH cells in the *pars intermedia* of hamster. Strikingly however, these cells do not show modifications after adrenalectomy in hamster. In *M. platythrix* the ACTH cells of the *pars distalis* and the type II cells of *pars intermedia*, share common histochemical characteristics, such as stronger affinity for fast green FCF, alcian blue at low pH and pyronin Y. In addition both these cell types are rich in alkaline phosphatase. On the basis of these findings it is suggested that the type II cells in the *pars intermedia* constitute an additional source of ACTH in this species.

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## Localization of 3-phosphoglyceric acid synthesis in the mother cell compartments and forespores of *Bacillus megaterium* and the effects of manganous ions on its metabolism

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**Abstract.** Rapidly metabolizable compounds such as glucose or glycerol were not utilized by *Bacillus megaterium* in the absence of manganese when grown in the supplemented nutrient broth medium. Under these conditions, growth ceased at low cell titre, 3-phosphoglyceric acid accumulated inside the cells and normal sporulation process was arrested. Addition of manganese to the medium caused disappearance of 3-phosphoglyceric acid, growth resumed and normal sporulation was observed. Synthesis of 3-phosphoglyceric acid occurred only in the mother cell compartments and it was transported for accumulation inside the forespores of *Bacillus megaterium* when grown in supplemented nutrient broth medium. Incubation of forespores in the presence of glucose or glycerol had no effect on 3-phosphoglyceric acid synthesis/accumulation, but it was completely utilized when forespores were incubated with manganese plus ionophore (X 537A). No other metal(s) could substitute for manganese suggesting that manganese plays crucial role in 3-phosphoglyceric acid metabolism.

**Keywords.** *Bacillus megaterium*; 3-phosphoglyceric acid; dipicolinic acid; germination; sporulation; ionophore.

### Introduction

The processes of sporogenesis and spore germination in bacilli have been considered as an unique model for studying the mechanism of differentiation because of the relative simplicity and the ready application of biochemical and genetic analysis to this system (Singh, 1983). During late stationary phase two distinct intracellular compartments, namely mother cell and forespore are formed (Young and Fitz-James, 1959). The latter compartment is metabolically active before becoming a dormant spore (Church and Halvorson, 1957; Setlow and Kornberg, 1969). The most significant event during sporulation is the accumulation of 3-phosphoglyceric acid (3-PGA) in the forespore/dormant spore which is utilized during the first minutes of spore germination (Setlow and Kornberg, 1970; Singh *et al.*, 1977; Singh and Setlow, 1979b). Among metal ions, manganese has been shown to play an essential role during normal sporulation in bacilli (Charney *et al.*, 1951) and it is specifically required as a cofactor for

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Abbreviations used: 3-PGA, 3-Phosphoglyceric acid; DPA, dipicolinic acid; SNB, supplemented nutrient broth; buffer A, 0.6 M sucrose, 0.1 M potassium phosphate buffer (pH 7.0) and 16 mM MgSO<sub>4</sub>; HSF, heat stable forms.

phosphoglycerate mutase (Oh and Freese, 1976; Singh and Setlow, 1979a) and fructose-1, 6-diphosphatase (EC 3.13.11) (Opheim and Bernlohr, 1975).

In the present communication evidence is presented to show the significance of manganese in the metabolism of 3-PGA and utilization of catabolizable substrates such as glucose and glycerol during normal sporulation in *B. megaterium*. These results also indicate the possible site in the mother cell compartment for 3-PGA synthesis which is subsequently transported for accumulation in the developing forespore inside the sporulating cell.

## Materials and methods

### *Reagents and enzymes*

[ $^{14}\text{C}$ ](U)-Glucose, [ $^{14}\text{C}$ ](U)-glycerol and  $^{32}\text{Pi}(\text{H}_3\text{PO}_4)$  were purchased from New England Nuclear Corp., Boston, Massachusetts, USA. Phosphoglycerate mutase (EC 2.7.5.3) and enolase (EC 4.2.1.11) were purified from actively growing vegetative cells of *B. megaterium* (Singh and Setlow, 1978b; 1979a). ADP was freed from contaminating ATP by incubating with hexokinase (EC 2.7.1.1) and glucose with subsequent boiling. The ionophore (X 537A) (Pressman and de Guzman, 1975) was a gift from Dr. W. E. Scott (Hoffman La Roche) and stock solution (1 mg/ml) was prepared in ethanol. All other enzymes and chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

### *Organism, cultural conditions, germination studies and isolation of forespores*

*B. megaterium* QM B 1551 was obtained from Dr. Hillel S. Lovinson (US Army Development Command Center, Natick, Massachusetts, USA). The organism was grown at 30°C in supplemented nutrient broth (SNB) medium (Setlow, 1975) and spores harvested.

The method for the isolation of forespores is a modification of Ellar and Postgate (1974) procedure. Samples (50 ml) of sporulating cells were centrifuged (5 min, 10,000 g) and washed with 25 ml of warm (37°C) buffer A (0.6 M, sucrose; 0.1 M, potassium phosphate buffer (pH 7.0) and 16 mM of  $\text{MgSO}_4$ ). The cell pellet was suspended in 6 ml of buffer A. To this, lysozyme (10 mg) was added followed by incubation for 10 min at 37°C. The lysozyme treated cells were washed twice with cold buffer A. The cells were suspended in 6 ml of cold buffer A and sonicated (30 s to 1.5 min, maximum output of sonifier cell Disrupter, Heat systems-Ultra Sonics, Inc., New York, USA) to release forespores. The sample was centrifuged twice for 3 min at 7,000 g and 3 min at 6,000 g respectively. The final forespore pellet had a very low level of contamination with mother cells and cell debris as seen under phase contrast microscope. Whole cell was used to study 3-PGA synthesis in mother cell compartment which is whole cell minus forespore. Germination of dormant spores was carried in KBr solution (Singh and Setlow, 1979b).

### *Labelling and identification of 3-PGA*

The organism was grown in SNB medium containing 1  $\mu\text{Ci}$  of either glucose or

glycerol [ $^{14}\text{C}$ ] (U) or  $^{32}\text{Pi}$  per ml. To this, 20 mM glucose or glycerol was added when the culture reached 3.5–5.0 absorbance at 600 nm. At various time intervals, 5 ml culture was filtered through a membrane filter (0.45  $\mu\text{m}$ ) pore size (Milipore Corp., Bedford, Massachusetts, USA) and the filter was extracted with 2 ml ice cold formic acid (0.5 M). The extracts were chromatographed on a column of AG1- $\times$ 8 (chloride form; 100–200 mesh; 0.8 by 10 cm size) by elution with HCl (20 mM) at a flow rate of 1 ml/min and fractions (3 ml) were collected. A 2 ml portion of each fraction was dried in a scintillation vial and its radioactivity was determined using liquid scintillation counter (Packard Co., Downers Grove, Illinois, USA). A reference sample of 3-PGA (0.1  $\mu\text{mol}$ ) was also chromatographed under the same conditions and 0.1 ml of each fraction was assayed colorimetrically for 3-PGA (Bartlett, 1959). The enzymatic identification of 3-PGA was done using enzymatic coupling methods *i.e.* the formation of glycerolphosphate or lactate from 3-PGA (Czok, 1974).

#### *Extraction and determination of 3-PGA and DPA from sporulating cells, forespores and dormant spores*

Forespores were isolated at designated times (arrows in figure 1), sporulating cells collected at about the time when DPA concentration was less than 5% and dormant spores were prepared by growing bacterium in SNB medium and cleaned (Singh, 1982b). 3-PGA and dipicolinic acid (DPA) were extracted from freeze dried samples of forespores, sporulating cells and dormant spores (each from 50 ml culture) by boiling in 80% *n*-propanol (Setlow and Kornberg, 1970).

3-PGA was assayed using luciferase assay method (Singh and Setlow, 1979b) after conversion of 3-PGA to ATP using ADP, enolase, phosphoglycerate mutase and pyruvate kinase as reported earlier (Singh, 1982b). DPA was determined colorimetrically (Rotman and Fields, 1967). Heat stable forms (HSF) were evaluated by the formula proposed earlier (Singh, 1982a).

#### *Incubation of forespores*

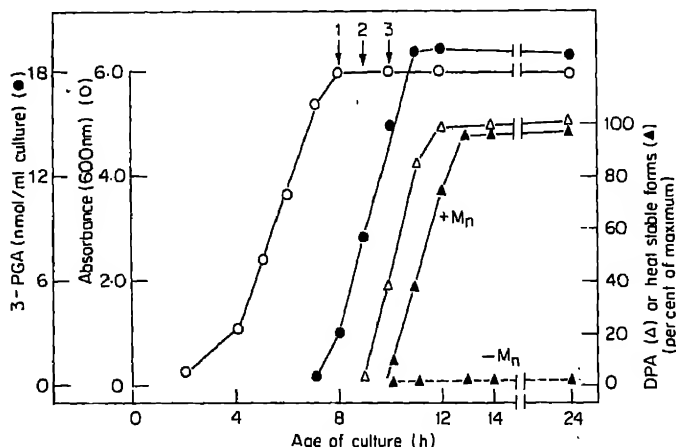
Forespores samples were incubated at 30°C with slow shaking (40 strokes/min) along with either glycerol or glucose (20 mM) in buffer A to determine *de novo* synthesis of 3-PGA. Sporulating cells or dormant spores were also used under similar conditions. At various intervals, samples were collected by centrifugation (5 min at 10,000 *g*), the pellet lyophilized, used for 3-PGA extraction and determination. In some experiments, ionophore dependent *in vivo* utilization of 3-PGA in the forespores was carried out in buffer A containing ionophore (10  $\mu\text{g/ml}$ ) plus manganese (1 mM) and samples were collected for 3-PGA estimation. The concentration of glucose in the growth medium was enzymatically determined (Gutman and Wahlefield, 1974) after cells had been removed from the culture by centrifugation.

## Results

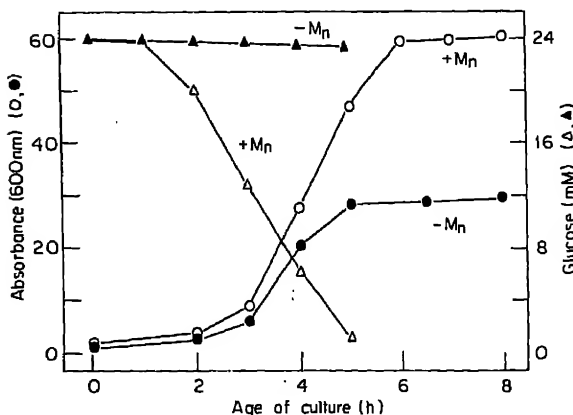
#### *Effects of manganese on growth and sporulation*

The organism grown in SNB medium accumulated 3-PGA about 1.5 h before DPA

accumulation followed by the appearance of heat stable forms (figure 1) when the concentration of manganese is about  $20\text{ }\mu\text{M}$ . In the absence of manganese, the organism failed to sporulate (1–3% spores when viewed under microscope), growth was considerably low (figure 2) and DPA synthesis/accumulation was not noticed (figure 1). The most interesting observation was the inability of the organism to utilize glucose in the absence of manganese while glucose was completely metabolised when the medium was supplemented with  $20\text{ }\mu\text{M}$  manganese (figure 2) which suggested that the supplementation of SNB medium with manganese and glucose greatly enhanced



**Figure 1.** Accumulation of 3-PGA, dipicolinic acid and development of heat stable forms during sporulation of *B. megaterium*. The organisms were grown in SNB medium in the absence or presence of manganese ( $20\text{ }\mu\text{M}$ ). The forespores were isolated at designated times (shown by arrows) as mentioned in the texts. 3-PGA, DPA and HSF were determined.



**Figure 2.** Growth and sporulation of *B. megaterium* in SNB medium plus glucose ( $20\text{ mM}$ ) in the presence or absence of manganese ( $20\text{ }\mu\text{M}$ ). Utilization of glucose from culture medium was done as mentioned in the texts.

growth and some of the spore specific events such as DPA synthesis and appearance of heat stable forms.

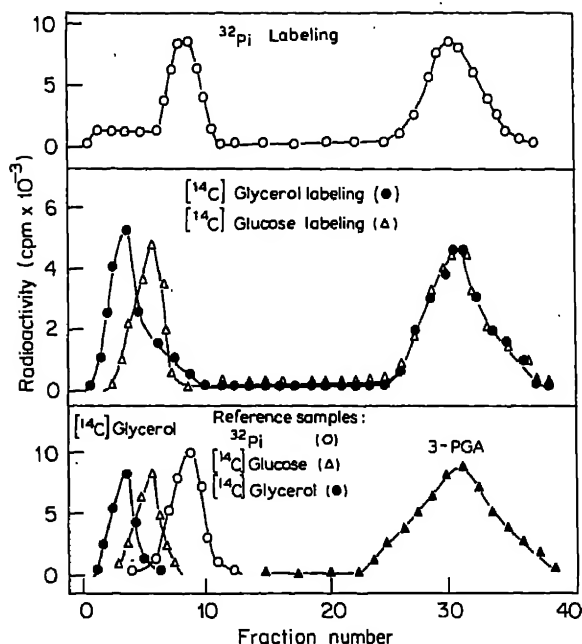
*Dependence of 3-PGA metabolism on manganese levels in the medium*

More than 95% of glucose or glycerol from the medium was metabolized when the organism was grown in SNB medium containing manganese (20  $\mu$ M) but these carbohydrates were not metabolized in the absence of manganese (figure 2) (data on glycerol utilization not given). To identify the metabolic block in the absence of manganese, the organism was grown in SNB medium containing  $^{32}\text{P}$ i (1  $\mu$ Ci), when the optical density reached 3.5, a sample was taken for 3-PGA extraction and 20 mM glycerol was added to the rest of the culture. Thirty min later, another sample was taken and 50  $\mu$ M of  $\text{MnCl}_2$  was added to the remaining medium. After another 30 min, a third sample was taken. Thin layer chromatography of cell extract in all the samples showed the accumulation of radioactive 3-PGA after glycerol addition which disappeared after manganese addition (data not shown). Also 3-PGA was further identified by isolating extracts from cells grown either in the presence of [ $^{14}\text{C}$ ] glycerol or [ $^{14}\text{C}$ ] glucose (1  $\mu$ Ci/ml) in the medium without manganese followed by Dowex-1-column chromatography of the extracts. A major peak appeared at the same position as the peak of authentic 3-PGA sample (figure 3).

The amount of 3-PGA that accumulated inside the cells and in the culture medium were also estimated (table 1). The intracellular concentrations of 3-PGA increased significantly when glucose (data with glucose not given) or glycerol was added in SNB medium in absence of manganese (table 2). Some 3-PGA also leaked out into the medium and its concentration did not change significantly even after manganese addition, whereas intracellular 3-PGA was no longer detectable in the cells.

Table 1. Accumulation of 3-PGA under various cultural conditions.

Growth condition at time of harvest			Amount of 3-PGA in:	
SNB medium plus	Growth conditions	$A_{600\text{nm}}$	Cells	Medium
			( $\mu\text{mol}/A_{600\text{nm}}$ )	( $\mu\text{M}$ )
Glucose (20 mM)	Exponential	3.5	1.2	< 0.2
	60 min after growth stopped	5.5	47.1	48.2
	30 min after $\text{Mn}^{2+}$ addition	5.9	1.2	48.0
Glucose (20 mM) + $\text{MnCl}_2$ (20 $\mu\text{M}$ )	Exponential	3.5	< 0.2	< 0.2
Glycerol (20 mM)	Exponential	3.5	1.2	0.2
	60 min after growth stopped	4.2	50.7	47.3
	30 min after subsequent $\text{Mn}^{2+}$ addition	5.1	1.2	48.0
Glycerol (20 mM) + $\text{MnCl}_2$ (20 $\mu\text{M}$ )	Exponential	3.5	< 0.2	< 0.2



**Figure 3.** Column chromatographic separation of [<sup>14</sup>C] or [<sup>32</sup>P] labelled compounds in cells. For labelling, 20 mM (U-<sup>14</sup>C)-glycerol or glucose or [<sup>32</sup>Pi] (1 μCi) was added to the culture in SNB medium and after 30 min incubation cells were extracted with formic acid. A column (0.8 × 12 cm) anion exchanger AG1- × 8 chloride (100–200 mesh) was charged with the extract and eluted with HCl (20 mM) at a flow rate of 1 ml/min as mentioned in the texts. A. Elution pattern of the [<sup>32</sup>P]-labelled extract. B. Elution pattern of the [<sup>14</sup>C] glycerol or glucose extract. C. Mixture of the [<sup>14</sup>C] or [<sup>32</sup>P]-labelled extracts and reference 3-PGA, all eluted from the same column.

**Table 2.** Effects of glycerol on 3-PGA accumulation in forespores and sporulating cells

SNB medium	Amount of 3-PGA (nmol/ml culture)	
	Before treatment	After treatment
Sporulating cells <sup>a</sup>	15.6	24.7
Forespores <sup>b</sup>		
Sample 1	11.5	10.3
Sample 2	17.2	14.8
Sample 3	20.1	18.2
Dormant spores <sup>c</sup>	21.6	21.7

<sup>a</sup> Sporulating cells were harvested at ( $A_{600\text{nm}} = 5.5$ ) washed twice and suspended in warm buffer A + glycerol (20 mM). 3-PGA was extracted before and after incubation (30 min) and assayed as described in the text.

<sup>b</sup> Forespores samples were prepared from the culture isolated at designated by arrows 1 to 3 in figure 1. Incubation etc. was done as stated above.

<sup>c</sup> Dormant spores were isolated from culture when > 95% free when viewed under phase contrast microscope.

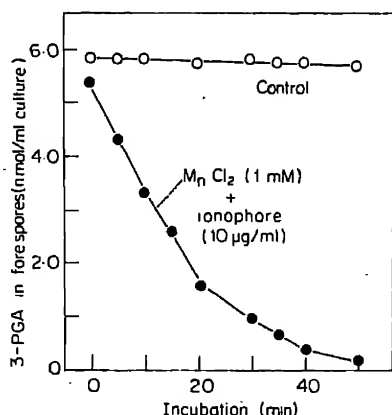
*Accumulation of 3-PGA in forespores and spores*

It has been well documented that 3-PGA is accumulated late during stages of sporulation *i.e.* about 1.5 h before the accumulation of DPA (figure 1). Previous work has shown that more than 90% of 3-PGA was accumulated within the forespores which are destined to form dormant spores but the site of 3-PGA synthesis whether mother cell compartment or forespore was not determined (Singh *et al.*, 1977). Results presented in table 2 indicated that forespores (containing about 5–10% DPA) when incubated with either glycerol or glucose failed to synthesize 3-PGA while sporulating cells isolated at the corresponding times were able to synthesize significant amount of 3-PGA and transported for accumulation inside the developing forespores (table 2). Under these experimental conditions, mother cell compartment represents whole cell (sporulating cell minus forespore). These data clearly demonstrated mother cell compartment as the site of 3-PGA synthesis. Interestingly, the intracellular concentrations of 3-PGA in the sporulating cells or forespore could not be increased once an initial 3-PGA (21 nmol) level was attained (table 2), though synthesis of 3-PGA was observed which was excreted out in the medium.

*In vivo utilization of 3-PGA in the forespores and dormant spores*

Forespores prepared at the designated times (arrows in figure 1) have stable 3-PGA pool (table 3) which suggested that one of the enzymes needed for its catabolism may not be fully active. Earlier studies (Singh and Setlow, 1978a; 1979b) have shown that the phosphoglycerate mutase requires manganese as a cofactor and was inactive in forespores and dormant spores. Results presented in figure 4, showed that the intracellular level of 3-PGA was constant when forespores were incubated with or without manganese or ionophore alone (data not shown) but 3-PGA level declined rapidly when forespores were incubated with manganese plus ionophore (figure 4). As expected manganese alone cannot cross the permeability barrier of forespore membrane and is facilitated by the ionophore.

Earlier studies have demonstrated that the 3-PGA was utilized to produce ATP and NADH needed during early phases of spore germination (Setlow and Setlow, 1977; Singh *et al.*, 1977). Incubation of dormant spores with manganese plus ionophore has



**Figure 4.** Effect of ionophore plus manganese on 3-PGA levels in isolated forespores. Forespores isolated when containing about 60% of the maximum amount of 3-PGA were incubated at 30°C in buffer A. The control represents incubation with either ionophore or manganese alone. 3-PGA extracted and analysed as described in the texts.



no effect on 3-PGA utilization (data not shown) probably because the dormant spore has two distinct membranes namely, cortex and plasma membrane which this ionophore probably failed to penetrate. During the germination of dormant spores in salt solution (KBr), 3-PGA was completely metabolised within 30 min (table 3). Addition of NaF (10 mM) arrested 3-PGA utilization under these conditions.

Table 3. Levels of 3-PGA during various stages of growth.

Stage of growth	3-PGA [n mol/mg (dry wt)]
Sporulating cells <sup>a</sup>	15.6
Dormant spores	21.8
Spores germinated for 30 min	1.6
Spores germinated for 30 min in the presence of NaF (10 mM)	16.2

Cells or spores were extracted and the extracts were passed through Norit column to remove nucleotides before 3-PGA assaying.

<sup>a</sup> Harvested at the point noted by arrow No. 2 in figure 1.

## Discussion

A large amount of 3-PGA was accumulated during late stages of sporulation, about 1.5 h before DPA synthesis (figure 1). This 3-PGA pool was stable in both isolated forespores and dormant spores inspite of the presence of all the necessary enzymes needed for its catabolism namely, phosphoglycerate mutase, enolase and pyruvate kinase (Singh *et al.*, 1977; Singh and Setlow, 1978a, 1979b). Since 3-PGA accumulation occurred in developing forespores even though the enzymes for 3-PGA catabolism were detected in extracts (forespore or spore) at levels similar to those in growing cells or germinated spores (Singh *et al.*, 1977), at least one of these enzymes must have had very little activity *in vivo* and was activated upon spore germination. 3-PGA was completely metabolised during first minutes of spore germination to generate much of the ATP and NADH needed for out growth (Setlow and Setlow, 1977; Setlow and Kornberg, 1970). As expected, 3-PGA utilization was completely inhibited when germination was carried out in KBr medium containing NaF suggesting that the 3-PGA catabolism occurred in the forward direction of glycolytic pathway *i.e.* 3-PGA to pyruvate as NaF is a known inhibitor of enolase (Singh and Setlow, 1978b). It cannot be ruled out that the slight decrease in 3-PGA levels in germinated spores in the presence of NaF may be due to the operation of other pathways not involving enolase.

Earlier studies have shown that phosphoglycerate mutase was inactive in the forespores and dormant spores and was activated upon spore germination (Oh and Freese, 1976; Singh and Setlow, 1978b, 1979a). This enzyme specifically requires manganese (Watabe and Freese, 1979) as a cofactor and not 2,3-diphosphoglycerate as reported in the literature (Grisolia and Carreras, 1975). Recently it was proposed that free manganous ions are chelated under *in vivo* conditions by a small molecular weight protein which was synthesized at about the same time that 3-PGA starts accumulating in the developing forespores (Singh, 1982b). These

observations suggest that the intracellular levels of free manganous ions play a significant role in 3-PGA metabolism when the organism was grown under various culture conditions (table 2). Similar observations were reported by Oh and Freese (1976) using various mutants of *B. subtilis*. Our earlier data on manganese uptake by the bacterium suggested that the sporulating cells were able to accumulate more than 95% manganese of the medium. About 70% of the total manganese present in dormant spores was released along with DPA and other smaller spore integuments during germination and the remaining 30% manganese could be bound loosely to spore components as evident from the manganese exchange data (Singh and Setlow, 1979b). Most interesting observations were the *in vivo* utilization of 3-PGA when the forespores were incubated with ionophore plus manganese (figure 4). These data support the earlier suggestions that the phosphoglycerate mutase was inactivated due to nonavailability of free manganous ions and could be activated *in vivo* upon incubation of forespores with ionophore plus manganese, suggesting that the free manganese was not available to the enzyme in sufficient amount.

Experiments were also done to determine the site of 3-PGA synthesis *i.e.* mother cell compartment and/or forespore. Results indicated that the incubation of forespore samples either with glycerol or glucose (data not shown) has no effect on 3-PGA synthesis (table 2). On the other hand, in the incubation of sporulating cells which represents mother cell compartment plus forespore under these conditions, there was a significant increase in intracellular 3-PGA pools suggesting that the 3-PGA was synthesized in the mother cell compartment and transported for accumulation in the forespores.

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## Studies on the effects of *in vitro* methylation on aminoacylation of transfer RNA

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**Abstract.** *In vitro* methylation of *Escherichia coli* transfer ribonucleic acid by cell free extracts of *Mycobacterium smegmatis* leads exclusively to the formation of 1-methyl adenine [Vani, B. R., Ramakrishnan, T., Taya, Y., Noguchi, S., Yamaiuzumi, Z. and Nishimura, S. (1978) *J. Bact.*, 137, 1085]. We have studied the effect of this modification on aminoacylation of *Escherichia coli* tRNA by mycobacterial enzymes. Aminoacylation with total algal protein hydrolysate as well as several individual aminoacids like methionine, valine, tyrosine, aspartic acid and lysine were monitored. In all the cases methylation had a positive effect on the extent of aminoacylation by mycobacterial enzymes. Decreased aminoacylation *in vitro* was observed when hypomethylated transfer RNA from ethionine treated cells was used as the substrate for aminoacylation.

**Keywords.** Transfer RNA; 1-methyladenine; methylation; aminoacylation.

### Introduction

Transfer RNA is uniquely abundant in modified bases. The effect of base modifications, specially methylation, has been studied in two ways: namely, by using hypomethylated tRNA either from mutants or from methylation inhibited cells, and by using hypermethylated tRNA obtained on chemical methylation using chemicals like dimethylsulphate (Geftter and Russell, 1969; Miller *et al.*, 1976). However this aspect has been elusive to any kind of generalisations.

Methylation is known to affect tRNA functions like aminoacylation and ribosome binding in certain systems (Hoburg *et al.*, 1979). Using ethionine fed rats, Ginzburg *et al.* (1979) have shown that hypomethylation of tRNA leads to decreased aminoacylation. Base modification also has been invoked in regulation of operons (Singer *et al.*, 1972; Bjork, 1980; Pope *et al.*, 1978).

The influence of the base modification can be directly on the structural interactions within the tRNA molecule leading to a change in its ability for aminoacylation. The role of tertiary structure of tRNA in aminoacylation is well appreciated and it is suggested that a conformational adaptation of tRNA and the enzyme on interaction could be a rate limiting step in aminoacylation (for a review see Schimmel, 1979). A large

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Abbreviation used: Buffer A, 10 mM Tris-HCl pH 7.6, containing 10 mM MgCl<sub>2</sub>, 6 mM 2-mercaptoethanol, and 10% glycerol.

proportion of modified bases being in the loop or the stem junction region, are thus involved in tertiary structure maintenance.

*Mycobacterium smegmatis* and *Escherichia coli* differ distinctly in the content of 1-methyl adenine in tRNA, the former being abundant and the latter lacking this modification. Further, we have also shown that cell free extracts of *M. smegmatis* form only 1-methyl adenine in *E. coli* tRNA, as demonstrated by chromatography of enzymatic digests of the *in vitro* reaction products (Vani *et al.*, 1978). Hence, we have attempted to study the influence of this modification, on the aminoacylation of tRNA mixtures from these two sources.

### Materials and methods

*Mycobacterium smegmatis* (SN<sub>2</sub>) was obtained from Dr. R. Bonicke, Institute for experimental Biology and Medicine, Borstel, FRG.

*Saccharomyces cerevisiae* var. *ellipsoideus* was obtained from Dr. G. Ramananda Rao of this laboratory.

*E. coli* tRNA, cold S-adenosyl methionine, Trizma base, Cacodylate, DEAE-cellulose, ethionine were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

Radioactive amino acids were obtained from Amersham, England with the given specific activity. [<sup>3</sup>H]-Tyrosine-189 mCi/mmol, [<sup>3</sup>H]-aspartic acid 217 mCi/mmol, [<sup>3</sup>H]-phenylalanine 1 Ci/mmol, [<sup>14</sup>C]-leucine-338 mCi/mmol, [<sup>14</sup>C]-asparagine-200 mCi/mmol, [<sup>14</sup>C]-lysine-336 mCi/mmol, [<sup>14</sup>C]-algal protein hydrolysate-54 mCi/mA and [<sup>3</sup>H]-methionine 290 mCi/mmol. All other chemicals used were of analytical grade.

*M. smegmatis* and *E. coli* were grown as described earlier (Vani *et al.*, 1979).

### Buffer A

10 mM Tris(hydroxymethyl)-aminomethane (Tris)-HCl pH 7.6 containing 10 mM MgCl<sub>2</sub>, 6 mM β-mercapto ethanol and 10% glycerol.

### In vitro methylation

The assay mixture (125 μl) contained 25 mM Tris-HCl pH 8.0 at 37°C, 20 mM Magnesium acetate, 9 μM S-adenosyl methionine (unlabelled), partially purified enzyme amounting to 200–300 μg protein and 1–2 absorbance units of tRNA. The reaction was carried out at 37°C for 30 min. The cell free extract fractionated on DEAE-cellulose column as 0.4 M KCl eluate was used as the enzyme source after dialysing out the KCl. The reaction was scaled up as per the requirement and methylated tRNA was extracted with phenol and precipitated with ethanol. The tRNA precipitate was collected by centrifugation, washed, dissolved and dialysed against buffer A and used in aminoacylation.

Aminoacylation was carried out as described by Deobagkar and Gopinathan (1979). In a total volume of 125 μl, the reaction mixture contained 100 mM Tris-HCl, pH 7.0 at 37°C, 10 mM Magnesium acetate, 20 mM KCl, labelled amino acid (25,000 cpm), 50 mM ATP, 1–2 A<sub>260</sub> units of tRNA and 100–200 μg protein, containing partially purified enzyme fraction (as in methylation reaction). The reaction was carried out for

specified time intervals at 37°C. After the incubation, a 50 or 100  $\mu$ l aliquot of the reaction mixture was spotted on Whatman No. 3 filter discs, dried under heat lamp, soaked in 10% trichloroacetic acid for 10 min, then washed thrice for 5 min each with 5% trichloroacetic acid followed by ethanol:ether mixture (1:1) and finally with ether. Then the discs were dried and counted in a Beckman LS-100 scintillation spectrometer.

## Results

### Effect on aminoacylation

The tRNA from *M. smegmatis* and yeast were studied as examples of m<sup>1</sup>A containing transfer RNA species and that of *E. coli* as a model for m<sup>1</sup>A deficient tRNA.

The charging efficiency of synthetases from the three sources namely *M. smegmatis*, *E. coli* and yeast, with heterologous tRNA as the substrate was compared with the activity of homologous tRNA. As table 1 shows for *E. coli* ligases tRNAs from *M. smegmatis* and yeast can serve as substrates only 50% as efficiently as the homologous tRNA, *M. smegmatis* ligases use *E. coli* tRNA more efficiently than homologous tRNA in this assay system. This is in confirmation with previous observation from our laboratory (Deobagkar, 1975). On the other hand, *M. smegmatis* ligases charge the yeast tRNA only around 50% as efficiently as the homologous tRNA. For yeast ligases *M. smegmatis* tRNA is better than *E. coli* tRNA as a substrate.

Table 1. Heterologous aminoacylation with [<sup>14</sup>C]-algal protein hydrolysate.

Ligases	tRNA	Activity Cpm/100 $\mu$ g tRNA	Percentage* activity
<i>E. coli</i>	<i>E. coli</i>	847	100
	<i>M. smegmatis</i>	421	50
	Yeast	368	44
<i>M. smegmatis</i>	<i>M. smegmatis</i>	335	100
	<i>E. coli</i>	371	110
	Yeast	192	57
Yeast	Yeast	646	100
	<i>M. smegmatis</i>	448	69
	<i>E. coli</i>	378	58

\* Calculated as percentage of homologous charging which is taken as 100.

In order to see whether the additional modification on *E. coli* tRNA by *M. smegmatis* methylases has any effect on its already efficient charging, *in vitro* methylated *E. coli* tRNA was used as substrate for *M. smegmatis* ligases. *In vitro* methylation of *E. coli* tRNA was carried out as described under methods, using partially purified methylases from *M. smegmatis*. The activity of tRNA methylase itself was checked by using [<sup>14</sup>CH<sub>3</sub>]-labelled S-adenosyl methionine. For aminoacylation, *E. coli* tRNA methylated with cold S-adenosyl methionine was re-extracted from *in vitro* reaction mixtures

and used in aminoacylation assays after extensive dialysis to remove phenol and the degradative products. Table 2 shows the results of such an experiment using [ $^{14}\text{C}$ ]-algal protein hydrolysate. It is observed that the complementary modification between *E. coli* and *M. smegmatis* improves their substrate capacity for the heterologous enzymes—by 2 fold in both the cases.

Table 2. Effect of methylation on aminoacylation with algal protein hydrolysate

Ligases	tRNA	Activity Cpm/100 $\mu\text{g}$ tRNA	Percentage* activity
<i>E. coli</i>	<i>E. coli</i> (native)	410	100
	<i>E. coli</i> (methylated)	434	105
	<i>M. smegmatis</i> (native)	312	76
	<i>M. smegmatis</i> (methylated)	814	198
<i>M. smegmatis</i>	<i>M. smegmatis</i> (native)	636	100
	<i>M. smegmatis</i> (methylated)	560	88
	<i>E. coli</i> (native)	874	137
	<i>E. coli</i> (methylated)	1802	283

\* Calculated as percentage of homologous charging which is taken as 100.

We have observed that ethionine inhibits growth as well as methylation of tRNA in *M. smegmatis*. At a concentration of 0.1% it inhibits growth upto 40% and the incorporation of [ $^{14}\text{CH}_3$ ]-group (of [ $^{14}\text{CH}_3$ ]-methionine) into tRNA to an extent of 90% indicating inhibition of methylation (Vani, 1982). Thus by growing *M. smegmatis* in the presence of ethionine one could get undermethylated tRNA. This tRNA is hypomethylated with respect to all the methylated bases, as the methyl group incorporation into tRNA is inhibited upto 90%. The tRNA after extraction from ethionine treated cells and purification on DEAE-cellulose column, was extensively dialysed against buffer A, ensuring that the preparation is free of ethionine. When this tRNA was compared with fully methylated tRNA from *M. smegmatis* for the extent of aminoacylation by *M. smegmatis* synthetases, it was seen that with [ $^{14}\text{C}$ ]-algal protein hydrolysate, the hypomethylated tRNA is a poor substrate for the reaction (figure 1).

Further, the native and the *in vitro* methylated tRNA of *E. coli* were compared with respect to their charging capacity with individual amino acids like tritiated tyrosine, aspartic acid, methionine and [ $^{14}\text{C}$ ]-labelled lysine. In these assays the synthetases were from *M. smegmatis*, as shown in figures 2, 3 and 4, the methylated tRNA was a better substrate in all the cases. In the case of tyrosine charging there is a 25% increase and in the case of aspartic acid it is about 54% increase over homologous charging. It is a two fold increase in the charging of methionine and a six fold in the case of lysine. Thus there is a quantitative difference in the efficiency of aminoacylation between the various tRNA species in response to methylation.

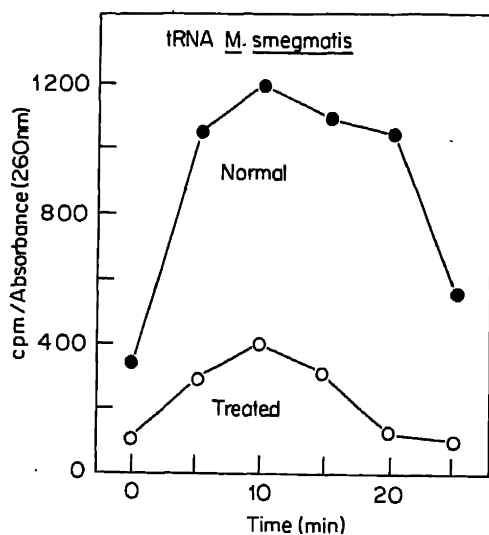


Figure 1. Aminoacylation of the total tRNA of *M. smegmatis* normal and that grown in presence of 0.1% DL-ethionine, with [ $^{14}\text{C}$ ]-algal protein hydrolysate. Activity has been expressed as radioactivity incorporated per one absorbance unit of tRNA—protein concentration being the same in all the assays.

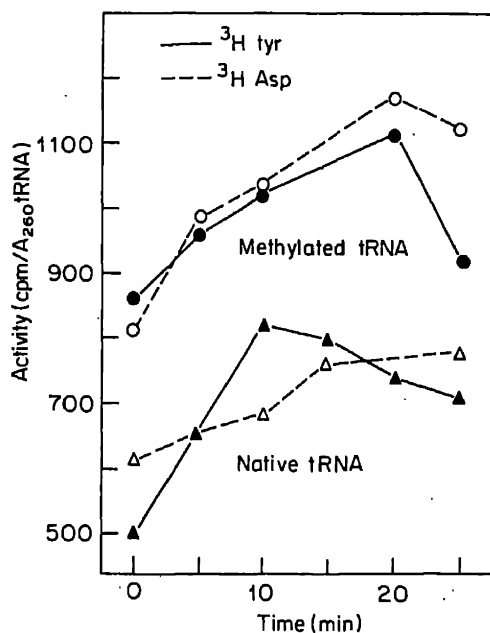


Figure 2. Time dependent charging of native and *in vitro* methylated total tRNA of *E. coli* with tritiated tyrosine and aspartic acid.



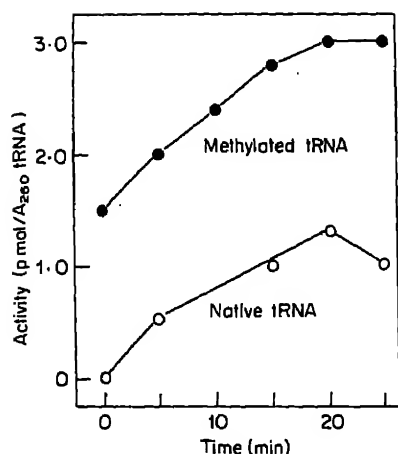


Figure 3. Aminoacylation of native and *in vitro* methylated total tRNA of *E. coli* with tritiated methionine.

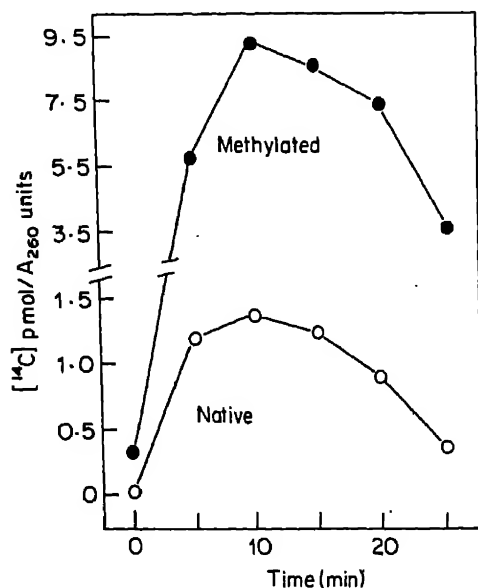


Figure 4. Aminoacylation of *in vitro* methylated and native total tRNA of *E. coli* with [<sup>14</sup>C]-labelled lysine.

## Discussion

In the tRNA structure, of about 22 bases present in the loop region, upto 20 are involved in tertiary interactions (Kim 1979), pointing to the importance of the tertiary structure of tRNA. There are several contradicting evidences as to the role of the base modification in aminoacylation. But these can be summarised to a certain extent to indicate that modified tRNA is a better substrate in heterologous aminoacylation

compared to the unmodified counterpart (Peterkofsky, 1964; Ginzburg *et al.*, 1979).

The earlier studies had been done with hypomethylated tRNA formed during methionine starvation or by treatment with ethionine. The present study has been confined to a particular modification *viz*, the methylation of adenine residue(s). It is interesting to note that the results presented here point to a similar conclusion, wherein methylated tRNA of *E. coli* serve as better substrates for aminoacyl tRNA synthetases of *M. smegmatis*. *E. coli* ligases aminoacylate the tRNA of yeast and *M. smegmatis* to a lesser extent (50%) compared with its own tRNA. Yeast ligases use *M. smegmatis* tRNA better than *E. coli* tRNA as substrate. *E. coli* tRNA which is as such a good substrate for *M. smegmatis* ligases becomes an even better substrate after *in vitro* methylation. However, ligases of *M. smegmatis* utilize yeast tRNA only (50%) as efficiently as its own tRNA though both the tRNAs contain  $m^1A$ . This inefficiency could be arising from the inherent differences between prokaryotic and eukaryotic systems, which is reflected in the activity of yeast ligases also with tRNA from *M. smegmatis* containing 1-methyl adenine. It is known from our studies that the only modification affected by *M. smegmatis* methylases, on *E. coli* tRNA is  $m^1A$  formation (Vani *et al.*, 1979). Further we have also demonstrated the presence in *M. smegmatis* of two activities of 1-methyladenine tRNA methyl transferase having different substrate specificity (Vani and Ramakrishnan, 1984). It is known from our sequencing studies (Vani *et al.*, 1984) that  $m^1A$  occurs at the 58th position in the cloverleaf structure. In the tertiary conformation, A-58 is involved in Hoogsteen base pairing with U-54, facilitating a sharp bend of the molecule in its three dimensional structure (Rich and Rajbhandary, 1976). In the light of the fact that one of the rate limiting steps in aminoacylation reaction is the conformational adaptation of both tRNA as well the cognate synthetase (Schimmel, 1979), the dynamic tertiary structure of tRNA could be playing an important role. It is interesting to note that  $m^1A$ -nucleotide can only form a Hoogsteen base pairing whereas adenine can involve in both Watson-crick as well as Hoogsteen base pairing with the uridine residue. In the rather sharp bending of the T $\psi$ C loop of tRNA in the tertiary structure there is also a hydrogen bonding between  $N_4$  of  $G_{61}$  and the phosphate of A-58. Further  $U_{54} \cdot A_{58}$  pair stacks over  $C_{61} \cdot G_{53}$  pair thus stabilizing the structure. These bonding and stacking interactions can be affected by base modification in the dynamic tertiary structure of tRNA and hence the function. Studies with purified single species tRNA would be more revealing. However, it is interesting to note that by proton NMR studies with *E. coli* tRNA<sup>Met</sup>, it has been possible to show the correlation between stability of tRNA structure and the modification of  $U_{54}$  residue (Davanloo *et al.*, 1979).

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## An *in vitro* test using cholesterol metabolism of macrophages to determine drug sensitivity and resistance of *Mycobacterium leprae*

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**Abstract.** Macrophages that have ingested live *Mycobacterium leprae* show a preferential accumulation of cholesterol ester. Such an accumulation is not seen, on the ingestion of dead bacteria. Among the macrophages that ingest live *Mycobacterium leprae*, the presence of dapsone or rifampicin prevents largely the alteration in the anticipated increase in the cholesterol ester indicating the sensitivity of the bacteria to the drug. In the small number of relapsed patients, the presence of dapsone did not reduce the cholesterol ester increase, suggesting that the *Mycobacterium leprae* present are either resistant or escaped detection. The method provides a rapid drug screening system for anti-*Mycobacterium leprae* activity of known and unknown compounds.

**Keywords.** *Mycobacterium leprae*; cholesterol; cholesterol ester; macrophage; drug sensitivity/resistance.

### Introduction

The screening of antimicrobials for anti leprosy activity has not been extensively undertaken. The mouse foot-pad growth technique with *Mycobacterium leprae* is claimed to be a well defined and reliable technique to test the viability and growth of *M. leprae* (Shepard, 1960). The same technique can be used to find out whether the *M. leprae* are susceptible to a drug or not by determining the growth in the foot-pad of mice, fed on a diet containing the drug (Shepard, 1967). This method has been refined and made more sensitive using thymectomised mice (Rees, 1966) or genetically bred nude mice (nu/nu) (Kohsaka *et al.*, 1978). However all these test systems need a minimum of 6-8 months even before preliminary assessment could be made. In view of this there is an urgent need for a rapid test system to determine drug sensitivity/resistance of *M. leprae*. The major limitation that has to be overcome with *M. leprae* is our inability to grow it *in vitro*, on any of the currently available bacteriological media in a reasonably short time.

We describe here a method for testing viability that could also be used for drug sensitivity testing of *M. leprae*, when the bacteria are resident in macrophages. The basic reaction monitored in this test system, is the influence that only live *M. leprae* exert on the cholesterol metabolism of the macrophages (Ishwari Kurup and

Mahadevan, 1982) leading to the formation of cholesterol ester from cholesterol taken up, as compared with macrophages with no live *M. leprae* inside. The present paper describes the adaptation of this observation for the development of a suitable and rapid drug sensitivity test that can even be used in an anti-leprosy drug screening programme.

## Materials and methods

*Mycobacterium leprae* were obtained from lepromatous tissue of bacillary positive, untreated patients or those under varying periods of chemotherapy. Bacillary suspensions were prepared (Ambrose *et al.*, 1974) and shown to be acid-fast and free from other contaminating bacteria. These fail to grow in normal mycobacteriological media. The bacilli were counted and  $5 \times 10^6$  bacilli were added to each Leighton tube containing cultured macrophages. *M. leprae* obtained from infected armadillo tissue (supplied by Dr. E. Storrs, Florida, USA) were also used in some of the experiments.

### Macrophage cultures

Macrophages from Swiss white mice were obtained from the peritoneal cavity following injection of 5 ml Eagle's minimal essential medium + 20% inactivated human serum (AB blood group) into the cavity, after killing the animal by cervical dislocation immediately after the injection of the medium. The peritoneal fluid was collected after agitating the cavity, and 0.7 ml of the fluid was added to each Leighton tube. The macrophages obtained from the peritoneal fluid adhered to the Leighton tubes. The medium was changed every 24 h; to remove non adherent cells. After 3 days of such culturing, esterase positive phagocytic cells, were predominantly distributed as an uniform layer at the flat bottom of Leighton tubes. There were no contaminating neutrophils, and non-adherent lymphocytes were not present in any significant numbers. Such tubes were divided into 5 sets. One set received viable *M. leprae* ( $5 \times 10^6$ /Leighton tube) either from human biopsies or armadillo and another heat-killed *M. leprae* ( $5 \times 10^6$ /Leighton tube). The control sets did not receive the *M. leprae* inoculum. The level of *M. leprae* to be added had earlier been confirmed as a good dose to elicit clear response from the macrophages (Ishwari Kurup and Mahadevan 1982). After 24 h of phagocytosis of the bacteria, the excess bacilli were removed and the macrophages were incubated with [ $^3\text{H}$ ]-cholesterol. The experiments were also carried using [ $^{14}\text{C}$ ]-acetate as precursor. Some of the cultured macrophages were exposed to drugs like dapsone (di(4-aminophenyl)-sulphone (DDS) (Burroughs Wellcome Co., Bombay) at a concentration of 1  $\mu\text{g}$ /Leighton tube (1  $\mu\text{g}$ /0.7 ml of medium) or rifampicin (Sigma Chemicals Co., St. Louis, Missouri, USA) (10  $\mu\text{g}$ /ml) for 72 h before *M. leprae* were added. Some of the control macrophages were not infected with *M. leprae* and used to study the effect of the drug alone and cholesterol uptake on lipid synthesis. In the drug treated cultures after 24 h of phagocytosis excess bacilli was washed off and the macrophages were labelled with [ $^3\text{H}$ ]-cholesterol/[ $^{14}\text{C}$ ]-acetate and left for 4 days for studying the uptake and synthesis of the lipids in the presence of the drug.

[ $^3\text{H}$ ]-Cholesterol was obtained as crude tritiated product from Bhabha Atomic Research Centre, Bombay and purified by repeated thin layer chromatography and

0.16  $\mu\text{Ci}$  was used for the uptake studies in each Leighton tube. [ $^{14}\text{C}$ ]-Acetate (with sp. activity of 56.7  $\mu\text{Ci}/\text{m mol}$  from Bhabha Atomic Research Centre, Bombay) was added in each Leighton tube at a level of 0.5  $\mu\text{Ci}$ . Many of the other parameters have been optimised during our earlier work, that has been reported (Iswari Kurup and Mahadevan 1982).

#### *Extraction and separation of lipids*

Following incubation, the macrophages were scrapped off the surface of the glass, the cells were microscopically counted and the lipids were extracted according to Dole's method (Dole, 1956). Lipids were separated by thin-layer chromatography on 20  $\times$  20 cm plates coated with silica gel (Chemical division, Glaxo laboratory, Bombay), at room temperature using hexane: ether: acetic acid (80:20:1) as development solvent. Identification of the lipid spots was made by staining with iodine vapours, using for reference, standard lipids (Sigma Chemicals Co., St. Louis, Missouri, USA).

#### *Determination of incorporation into lipids*

After identification of the lipid spots, the silica gel corresponding to the position of each standard, from the experimental sample, was carefully scrapped into scintillation vials. This was done after the evaporation of iodine and full decolourization. Bray's scintillation fluid (10 ml) was added to each vial and its radioactivity determined by using Kontron MR-300 automatic scintillation counter.

Since each experiment is done with separate crop of macrophages from mice and bacteria derived from different patient materials, variability is a problem. This has been partially overcome with averaging the experimental values with indication of standard deviation and *P* values.

### Results

We had shown earlier that macrophages with or without *M. leprae* were able to incorporate [ $^{14}\text{C}$ ]-acetate into lipids and also take up cholesterol and convert a portion of it to cholesterol ester (Ishwari Kurup and Mahadevan, 1982).

When labelled cholesterol was used for uptake studies it was observed that the total uptake of cholesterol inside the macrophages was comparatively less when they were infected with *M. leprae*. Nevertheless, presence of *M. leprae*, induced increased level of cholesterol ester as seen by the higher ratio of labelled cholesterol ester to cholesterol in the culture, as compared with macrophages with no *M. leprae* (table 1). No such increase in the ester level was seen in macrophages exposed to autoclaved *M. leprae* (table 1). But if the macrophages had been exposed to the drug earlier and then *M. leprae* were added and the bacteria were exposed to such intracellular drug for 24 h before the cholesterol uptake during the next 4 days, then the results would be as shown in table 1. This showed that DDS was able to inactivate *M. leprae* from both armadillo as well as those derived from human leproma tissues. Due to this inactivation the cholesterol ester level was not increased and the ratio of cholesterol ester to cholesterol (as radioactivity associated with ester to that associated with cholesterol) was

Table 1. Cholesterol and cholesterol ester level in macrophages after incubation with [ $^3\text{H}$ ]-cholesterol in the presence and absence of *M. leprae* (as cpm/ $10^6$  macrophages).

Culture	Armadillo <i>M. leprae</i>				Human <i>M. leprae</i> (Biopsy) 411				Human <i>M. leprae</i> (Biopsy) 414			
	Cholesterol	Cholesterol ester	Ratio*	Cholesterol	Cholesterol ester	Ratio*	Cholesterol	Cholesterol ester	Cholesterol	Cholesterol ester	Ratio*	Cholesterol
Macrophage only	35782	3741	0.10	11331	304	0.027	15420	1600				
Macrophages + heat killed <i>M. leprae</i>	37071	5189	0.14	10501	422	0.04	13886	670				
Macrophage + live <i>M. leprae</i>	22344	5347	0.24	8320	980	0.12	9562	1912				
Macrophages + DDS only	38125	4685	0.12	11680	350	0.03	13467	537				
Macrophages + DDS + live <i>M. leprae</i>	36475	5280	0.14	11830	621	0.052	16700	665				

DDS-Diphenyl dithiopylone (dapson).

\* Ratio of cpm in cholesterol ester/cpm in cholesterol.

statistically similar to the control and/or heat killed *M. leprae* added macrophages. There was a lower level of cholesterol in macrophages, in live *M. leprae* added cultures as compared with others. This could be due to a decreased uptake of cholesterol since label is added after *M. leprae* addition or due to conversion of cholesterol to some other components, besides the ester. This is a noteworthy feature for future studies.

Table 2 presents data from a typical experiment using [ $^{14}\text{C}$ ]-acetate as a precursor to determine the level of cholesterol ester in the macrophages. Results are similar as seen in table 1, indicating the sensitivity of *M. leprae* to the drug DDS. The consolidated data of 9 such experiments are presented in table 3 and the statistically significant difference

**Table 2.** Incorporation of [ $^{14}\text{C}$ ]-acetate into cholesterol and cholesterol ester in macrophages (cpm/ $10^6$  macrophages) with and without human *M. leprae* and drug (biopsy No. 483).

	Macrophage	Macrophage + heat killed <i>M. leprae</i>	Macrophage + live <i>M. leprae</i>	Macrophage + DDS*	Macrophage + DDS* <i>M. leprae</i>
Cholesterol	3812	3516	1811	3350	3190
Cholesterol ester	1075	970	897	1250	977
Ratio of ester to cholesterol**	0.28	0.275	0.495	0.373	0.30

\* Dapsone (DDS).

\*\* Ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/ $10^6$  macrophages.

**Table 3.** Ratio of cholesterol ester/cholesterol during different treatments with different preparation of *M. leprae* (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/ $10^6$  macrophages).

<i>M. leprae</i> isolated from	Macrophage	Macrophage + heat killed <i>M. leprae</i>	Macrophage + live <i>M. leprae</i>	Macrophage + DDS (dapsone)	Macrophage + DDS + <i>M. leprae</i>
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
Armadillo tissue*	0.10	0.14	0.24	0.12	0.14
Armadillo tissue	0.03	0.01	0.19	0.02	0.07
Armadillo tissue	0.04	0.03	0.12	0.04	0.05
<i>Human M. leprae</i>					
Biopsy 411	0.03	0.04	0.12	0.03	0.05
414	0.10	0.05	0.20	0.04	0.04
417	0.18	0.19	0.32	0.18	0.14
426	0.27	0.12	0.67	0.22	0.20
483**	0.28	0.20	0.50	0.37	0.31
497**	0.18	0.14	0.39	0.19	0.19
Mean with S.D.	0.133 $\pm$ 0.09	0.102 $\pm$ 0.06	0.30 $\pm$ 0.17	0.123 $\pm$ 0.11	0.132 $\pm$ 0.08

\* Using labelled cholesterol.

\*\* Using labelled [ $^{14}\text{C}$ ]-acetate.

*a* - *b* *P* - (n.s.)

*c* - *a* *P* < 0.025

*e* - *b* *P* - (n.s.)

*e* - *a* *P* - (n.s.)

*d* - *e* *P* value > 0.5 (n.s.).



(by student 't' test) of the cholesterol ester level in macrophage containing live *M. leprae* and drug treated *M. leprae* is clear. It is noteworthy that the human biopsy derived *M. leprae* were from six different patients and the three armadillo derived *M. leprae* are from one infected tissue of armadillo. The value of ratio for each of the experimental conditions varied from experiment to experiment probably due to variability in the macrophage population. So a larger number of experiments will be needed in fully standardised conditions to narrow the difference and also do an analysis of variance. However, the same population of macrophage has consistent behaviour, as expected by the condition of experimentation. Thus, the data on the ratio is represented as an average of all experiments with the standard deviation under each category and *P* values determined for these average values for each comparable categories.

One basic requirement in any method that indicates the viability of *M. leprae* is that the parameter tested should vary with difference in the viability of the test bacterium. We could clearly demonstrate this using varying levels of *M. leprae* with constant amount of macrophages (table 4). This showed that, as viable *M. leprae* increased, the level of cholesterol ester was also augmented with increasing level of the ratio of cholesterol to its ester. This indicated that the phenomenon depended on the presence of viable bacteria and thus would reflect the viability of the test *M. leprae* as well. No linear relationship is seen, because, there could not be an uniform ratio of live to dead *M. leprae* in all aliquots, since *M. leprae* basically came from human tissues.

**Table 4.** Effect of increasing amount of *M. leprae* in the cholesterol metabolism of macrophages (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/ $10^6$  macrophages).

Amount of <i>M. leprae</i> added to $1 \times 10^6$ macrophages	Ratio of cholesterol ester to cholesterol
No. <i>M. leprae</i> added	0.10
$2.5 \times 10^6$ <i>M. leprae</i>	0.10
$3.0 \times 10^6$ <i>M. leprae</i>	0.11
$8 \times 10^6$ <i>M. leprae</i>	0.25
$10 \times 10^6$ <i>M. leprae</i>	0.50
$15 \times 10^6$ <i>M. leprae</i>	0.53

Recently the concentration of DDS within  $10^6$  macrophages when the cells were exposed to  $1 \mu\text{g}$  of DDS has been determined by Jagannathan and Mahadevan (personal communication). It is estimated as  $25 \text{ ng}/10^6$  macrophages and each macrophage is expected to have DDS to a level of  $1.7\text{--}12.5 \mu\text{g}/\text{ml}$ . The estimation was done by them using fluorometric measurement which has a sensitivity upto  $4 \text{ ng}$  level of DDS. The concentration obtained in the macrophages is much higher than the minimum inhibition concentration  $0.003 \mu\text{g}/\text{ml}$  needed to function as bacteriostatic to *M. leprae* as reported by Ellard *et al.* (1971) and Peters *et al.* (1975).

Experiments were also done using *M. leprae* derived from clinically relapsed patients and those suspected to harbour drug resistant bacteria in their bodies. In such experiments it was clear that *M. leprae* showed no sensitivity to the drug, dapsone, since

even in the presence of dapsone inside the macrophages, they behaved as if they were alive, by increasing the cholesterol ester level more than the control. The data in table 5 presents one such isolate (biopsy No. 499 using [ $^{14}\text{C}$ ]-acetate as labelled precursors) and table 6 presents a consolidated data on all the five separately isolated bacteria.

**Table 5.** Cholesterol and the ester level in macrophages exposed to "resistant" bacilli isolated from clinically relapsed patient during dapsone (DDS) treatment (cpm/ $10^6$  macrophages).

	Cholesterol	Cholesterol ester	Ratio*
Macrophage	2308	313	0.136
Macrophage + heat killed <i>M. leprae</i>	2541	432	0.17
Macrophage + DDS	2020	303	0.15
Macrophage + DDS + <i>M. leprae</i>	1550	687	0.44
Macrophage + <i>M. leprae</i>	2034	799	0.39

\*Ratio of radioactivity (cpm) is cholesterol ester to that in cholesterol/ $10^6$  macrophages.

**Table 6.** Ratio of cholesterol ester/cholesterol in macrophages exposed "resistant" bacteria during different conditions (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/ $10^6$  macrophages).

<i>M. leprae</i> from	Macrophage	Macrophage + heat killed <i>M. leprae</i>	Macrophage + live <i>M. leprae</i>	Macrophage + DDS (dapsone)	Macrophage + DDS + <i>M. leprae</i> (dapsone)
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
423*	0.14	0.11	0.35	0.12	0.57
435*	0.09	0.03	0.55	0.06	0.54
495**	0.18	0.16	0.49	0.19	0.57
499**	0.14	0.17	0.39	0.15	0.44
277**	0.14	0.12	0.30	0.15	0.61
Mean with S.D.	0.138 $\pm$ 0.029	0.118 $\pm$ 0.05	0.416 $\pm$ 0.091	0.134 $\pm$ 0.043	0.546 $\pm$ 0.057

\* Using labelled cholesterol.

\*\* Using labelled acetate.

*a* - *e*;  $P < 0.025$

*c* - *e*;  $P < 0.05$

(no. of experiments = 5).

Similar results were obtained with another drug rifampicin and with [ $^{14}\text{C}$ ]-acetate as precursors as shown in table 7 and it could be seen that of the 3 isolates tested all the 3 were sensitive to rifampicin.

Table 7. Ratio of cholesterol ester/cholesterol in macrophages during different conditions with rifampicin (ratio of radioactivity (cpm) in cholesterol ester to that in cholesterol/10<sup>6</sup> macrophages).

Type of <i>M. leprae</i>	Macrophage	Macrophage heat killed <i>M. leprae</i>	Macrophage + live <i>M. leprae</i>	Macrophage + Rifampicin	Macrophage + Rifampicin + live <i>M. leprae</i>
Armadillo Bacilli	0.30	0.44	0.93	0.47	0.45
Armadillo Bacilli	0.30	0.44	0.93	0.47	0.47
FMR 142 (original human biopsy bacteria)	0.28	0.20	0.65	0.22	0.32

## Confirmation by mouse foot-pad

Since the mouse foot-pad test is generally accepted as a reliable procedure for viability (Shepard, 1960, 1967) we confirmed the drug sensitivity/resistance of some isolates from the human biopsies by both mouse foot-pad method and the method described in this paper. Such isolates are FMR 423, 435, 277. The mouse foot-pad tests were carried out as per the procedures of Rees (1964) and Shepard (1967). All the three isolates tested and shown to be resistant to dapsone by the cholesterol uptake method were found to be resistant to the drug by growing in mice fed with varying concentration of the drug (tables 8 and 9).

**Table 8.** Correlation of resistance of *M. leprae* to drug (dapsone) by the *in vitro* method and mouse foot-pad experiment.

Bacteria isolated from biopsies	Present <i>in vitro</i> test resistance to DDS	Experiment No.	Resistance to drug DDS (mouse foot-pad growth)
FMR 423	Resistant	AH 227	Resistant (as indicated by growth at 9th month in drug fed animals)
FMR 435	Resistant	231	Resistant (see table 9)
FMR 277	Resistant	143	Resistant (as indicated by growth of <i>M. leprae</i> seen at 9th month drug fed animals)

**Table 9.** Mouse foot-pad growth of *M. leprae* from patient biopsy FMR 435 suspected to have drug resistant *M. leprae*.

Clinical assessment:- Treated case, possibly relapsed.			Inoculum: $10^4$ AFB/0.03 ml/H.F.P. Swiss white random bred mice BI = 5+ : MI = 3%
	Number of bacteria 7th month post inoculation	Number of bacteria 9th month post inoculation	
Untreated controls	$7 \times 10^5$ AFB/FP	$2 \times 10^5$ AFB/FP	
0.0001 mg % DDS*	$10 \times 10^5$ AFB/FP	$1.5 \times 10^5$ AFB/FP	
0.001 mg % DDS*	$12.5 \times 10^5$ AFB/FP	$1.5 \times 10^5$ AFB/FP	
0.01 mg % DDS*	$24 \times 10^5$ AFB/FP	$1.3 \times 10^5$ AFB/FP	

BI-Bacteriological index-total bacterial load.

MI-Morphological index-viable bacterial load.

AFB/FP-Acid fast bacilli per foot-pad.

\* Mice fed with indicated concentration of DDS.

PP-Foot-pad.

## Discussion

It is clear from our earlier data (Ishwari Kurup and Mahadevan, 1982) and from the present experiments that heat killed *M. leprae* have no ability to interact with macrophages of mice to allow increased accumulation of cholesterol ester. Using this information the viability of *M. leprae* in the presence of drug inside the macrophages could be assessed. This has been possible to do so, as illustrated from the data provided in tables 1 and 6 when DDS and rifampicin, respectively, were used as the drugs. Viability or otherwise in the presence of drug would be indicated by cholesterol ester accumulation and this in turn would indicate sensitivity/resistance of the *M. leprae* to the drug tested. This method is reliable and consistent as has also been shown by correlative information of the drug sensitivity resistance of the same *M. leprae* preparation as tested by mouse foot-pad technique.

A few other *in vitro* techniques have been described using uptake of radioactive dihydroxyphenylalanine (DOPA) by *M. leprae* as an indicator of viability (Ambrose *et al.*, 1978); incorporation of [ $^3\text{H}$ ]-thymidine by *M. leprae*, phagocytosed inside macrophages (Nath *et al.*, 1982 and Prasad *et al.*, 1981) or incorporation of [ $^{14}\text{C}$ ]-acetate into lipids of *M. leprae* while the bacteria are still in isolated human tissues (Lakshmi *et al.*, 1983). All these have been used with reasonable success for screening the viability and drug sensitivity of *M. leprae* from human biopsies. A major problem that has to be overcome with *M. leprae* is their inability to grow *in vitro* in any bacteriological medium in a short time. Thus one has to use some metabolic activity as an indicator of the non dividing bacteria for viability testing.

The advantages of the drug screening system described here is that it can use macrophages derived from mice and the readily available labelled compound [ $^{14}\text{C}$ ]-acetate. The test can be completed in 9 days time and the results are consistent thus far. Since metabolic compounds are isolated and the radioactivity (at appreciable levels) are estimated in these compounds, so greater confidence exists. By a clear demonstration of a metabolic trigger in the host cell by the live bacteria, we are able to assay the viability of non-dividing *M. leprae* in the *in vitro* system which is probably closer to what happened in an *in vivo* situation. As indicated in our earlier paper cholesterol ester accumulation has a significance to the foamy nature of host cells infected with *M. leprae*. Thus the process monitored is one that could be occurring in the pathological state of leprosy in the patient. These advantages clearly put this technique, perhaps, above all the other methods described so far.

Experiments for further standardization of the technique and adoption of the method to determine minimum inhibition concentration of the known drugs are in hand.

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## Purification and regulation of aspartate transcarbamylase from germinated mung bean (*Vigna radiata*) seedlings

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**Abstract.** Aspartate transcarbamylase (EC 2.1.3.2) was purified to homogeneity from germinated mung bean seedlings by treatment with carbamyl phosphate. The purified enzyme was a hexamer with a subunit molecular weight of 20,600. The enzyme exhibited multiple activity bands on polyacrylamide gel electrophoresis, which could be altered by treatment with carbamyl phosphate or UMP indicating that the enzyme was probably undergoing reversible association or dissociation in the presence of these effectors. The carbamyl phosphate stabilized enzyme did not exhibit positive homotropic interactions with carbamyl phosphate and hysteresis. The enzyme which had not been exposed to carbamyl phosphate showed a decrease in specific activity with a change in the concentration of both carbamyl phosphate and protein. The carbamyl phosphate saturation and UMP inhibition patterns were complex with a maximum and a plateau region. The partially purified enzyme also exhibited hysteresis and the hysteric response, a function of protein concentration, was abolished by preincubation with carbamyl phosphate and enhanced by preincubation with UMP. All these observations are compatible with a postulation that the enzyme activity may be regulated by slow reversible association–dissociation dependent on the interaction with allosteric ligands.

**Keywords.** Aspartate transcarbamylase; mung bean; purification; regulation; hysteresis; association–dissociation.

### Introduction

Aspartate transcarbamylase (EC 2.1.3.2) catalyzes the carbamylation of L-aspartate to N-carbamyl L-aspartate by carbamyl phosphate with the release of orthophosphate ( $P_i$ ). This is the first committed step in pyrimidine biosynthesis in microorganisms and plants (Bethell and Jones, 1969; Neumann and Jones, 1964). Although extensive studies were conducted with the enzyme from *Escherichia coli*, relatively little information is available on the enzyme from plant sources. The enzyme from wheatgerm (Yon, 1981) and mung bean seedlings (Achar *et al.*, 1974; Rao *et al.*, 1979) has been purified to homogeneity. Earlier methods of purification of the enzyme from mung bean yielded preparations which were unstable and hence only a few kinetic and regulatory properties were studied (Savithri *et al.*, 1978a,b). It was therefore necessary to standardize a purification procedure which yielded a stable enzyme preparation. This

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Abbreviations used:  $P_i$ , Orthophosphate; DEAE, diethylaminoethyl; GdmCl, guanidinium chloride, Buffer A, 50 mM sodium acetate-acetic acid buffer, pH 4.7 containing 6 M GdmCl;  $M_r$ , molecular weight; Tris, Tris-(hydroxymethyl) aminomethane; SDS, sodium dodecyl sulphate; PALA, N-phosphonoacetyl-L-aspartate.



paper describes a modified procedure for the purification of the enzyme and a probable mechanism for the regulation of the enzyme activity by slow reversible association-dissociation.

## Materials and methods

### Materials

All the chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, except diethyl aminoethyl-(DEAE)-cellulose (DE-52) which was obtained from Whatman Ltd., Maidstone, Kent, UK. Mung bean seeds were purchased from the local market.

### Methods

Carbamyl phosphate was purified by the method of Gerhart and Pardee (1962).  $\omega$ -Aminoethyl-Sepharose was prepared by coupling 1,6-diaminohexane to cyanogen bromide activated Sepharose 4B (March *et al.*, 1974). Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Subunit molecular weight ( $M_s$ ) of the enzyme was determined by gel filtration on Sepharose 6B in 6 M guanidinium chloride (GdmCl) (Mann and Fish, 1972). Polyacrylamide gel electrophoresis (PAGE) was conducted according to Davis (1964).

### Estimation of the enzyme activity

The standard assay mixture (1 ml) contained 0.1 M Tris(hydroxymethyl)-aminomethane (Tris)-acetate buffer, pH 8.0, carbamyl phosphate (2.5 mM), L-aspartate (10 mM) was neutralised to pH 8.0 with NaOH and an appropriate amount of the enzyme. The reaction was started by the addition of L-aspartate unless otherwise indicated. After incubation for 20 min at 25°C, the reaction was terminated by the addition of 0.05 ml of 20% perchloric acid. The amount of N-carbamyl L-aspartate formed was estimated (Prescott and Jones, 1969). When partially purified enzyme preparations were used, the denatured protein after the addition of perchloric acid was removed by centrifugation at 1,000 *g* for 10 min before the addition of the reagents to develop the colour.

## Results

### *Isolation of aspartate transcarbamylase from germinated mung bean seedlings*

All the operations were carried out at 0–4°C. All centrifugations were performed in a Sorvall RC 5B refrigerated centrifuge at 10,000 *g* for the time intervals indicated.

### *Crude extract*

Mung bean (2.5 kg) seedlings germinated for 48 h were thoroughly washed with distilled water, chilled and blended in 4 batches with 200 ml each of 0.1 M Tris-acetate buffer, pH 8.0. The homogenate was passed through two layers of cheese-cloth and the filtrate was centrifuged for 30 min. The resulting supernatant was designated as crude

**Table 1.** Purification of aspartate transcarbamylase from mung bean (*Vigna radiata*) seedlings.

Fraction	Total protein (mg)	Total activity (Units*)	Specific activity (Units/mg)	Yield per cent	Fold purification
Crude extract	24752	40.8	0.00165	100	—
MnSO <sub>4</sub> supernatant	22400	40.6	0.00181	100	1
First (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	6103	25.4	0.0042	62	3
DEAE-Cellulose eluate	2162	22.7	0.0105	56	6
Second (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	819	12.6	0.0154	31	9
AH-Sepharose fractions	1.8	10.8	5.0	26	3636

\*  $\mu\text{mol N-carbamyl-L-aspartate}$  formed per min at pH 8.0 and 25°C.

extract (1820 ml, table 1). To the crude extract, MnSO<sub>4</sub> was added to a concentration of 10 mM and stirred for 30 min. The precipitated nucleoproteins were removed by centrifugation for 30 min (1750 ml, table 1). To the MnSO<sub>4</sub> supernatant, solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 45% saturation and the supernatant fraction was raised to 60% saturation by a further addition of solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitate obtained was dissolved in 0.01 M Tris-acetate buffer, pH 8.0 and desalted in 2 batches on a column (3.5 × 50 cm) of Sephadex G-25 previously equilibrated with the same buffer (193 ml, table 1). The desalted enzyme was loaded on to a DEAE-cellulose column (2 × 60 cm) equilibrated previously with 0.01 M Tris-acetate buffer, pH 8.0. The column was washed with 500 ml of 0.05 M KCl solution and the enzyme was eluted with 0.2 M KCl solution (198 ml, table 1). The enzyme was precipitated at 45–60% saturation and dissolved in 0.01 M potassium phosphate buffer, pH 7.6 and desalted on a column (2 × 50 cm) of Sephadex G-25 in the same buffer. The desalted enzyme (15 ml, table 1) was passed through an  $\omega$ -aminoethyl-Sepharose column (1 × 25 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.6 at a flow rate of 10 ml/h. The column was washed with 150 ml of the equilibrating buffer followed by 150 ml of the same buffer containing 0.05 M KCl. The enzyme was eluted with a linear gradient (100 ml) of 0.05–0.5 M KCl in 10 mM potassium phosphate buffer, pH 7.6. The eluting buffer also contained 2 mM carbamyl phosphate to stabilize the enzyme. Fractions (1 ml) were collected and the absorbance at 280 nm as well as enzyme activity of the fractions were determined. The fractions with high specific activity (< 6.0) were pooled and dialysed against 1 L of 10 mM potassium phosphate buffer, pH 7.6 with 3–4 changes. The dialysed enzyme (8 ml, table 1) was centrifuged at 15,000 *g* for 10 min and stored frozen at –40°C in 1 ml aliquots.

The enzyme obtained by this method had a specific activity of ~ 6.0 which was the highest reported so far for this enzyme from mung bean seedlings (Ong and Jackson, 1972; Achar *et al.*, 1974; Rao *et al.*, 1979). The results of a typical purification procedure are shown in table 1. The recovery of the enzyme was about 26% with 3600 fold purification.

### *Preparation of the partially purified enzyme*

For some of the studies reported in this paper, a partially purified enzyme preparation was used. The second ammonium sulphate (table 1) precipitate was dissolved in 20 mM Tris-acetate buffer, pH 8.0 and desalted on a small column (1.5 × 15 cm) of Sephadex G-25 using the same buffer. The enzyme was used immediately. Control experiments showed that the enzyme retained complete activity for 2 h under these conditions. It was devoid of contaminating activities like carbamyl phosphate synthetase (EC 2.7.2.5), aspartokinase (EC 2.7.2.4), aspartate aminotransferase (EC 2.6.1.1) and phosphatase (EC 3.1.3.1). Its specific activity varied between 0.01–0.016.

One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1  $\mu$ mol of N-carbamyl-L-aspartate per min at 25°C and pH 8.0. Specific activity was defined as units per mg of protein.

### *Physicochemical properties of the enzyme*

**Polyacrylamide gel electrophoresis:** The purified enzyme showed multiple bands of enzyme activity (figure 1A) on PAGE and these corresponded with the protein bands (figure 1B). No additional protein bands were located on the enzyme when stained with Coomassie brilliant blue. The nature of these multiple bands is discussed later. As different staining procedures were used to locate enzyme activity and protein bands, the correspondence between the protein bands and activity bands was not very good.

**Molecular weight of the enzyme:** The  $M_r$  of the enzyme was determined by gel filtration on Sephacryl S-200 (figure 1D) to be 125,000.

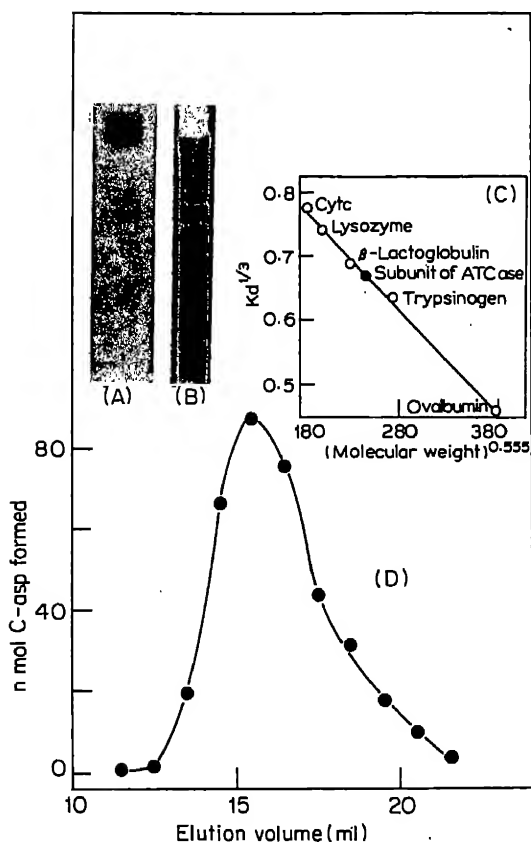
**Molecular weight of the subunit:** The enzyme could not be denatured completely by sodium dodecyl sulphate (SDS) even after extensive boiling in the presence of 2-mercaptoethanol. Hence, the subunit  $M_r$  of the enzyme was determined by gel filtration on sepharose 6B in the presence of 6 M GdmCl. The enzyme gave a single symmetrical peak and from a standard graph (figure 1C), the  $M_r$  of the subunit was calculated to be 20,600.

### *Regulatory properties of the purified enzyme*

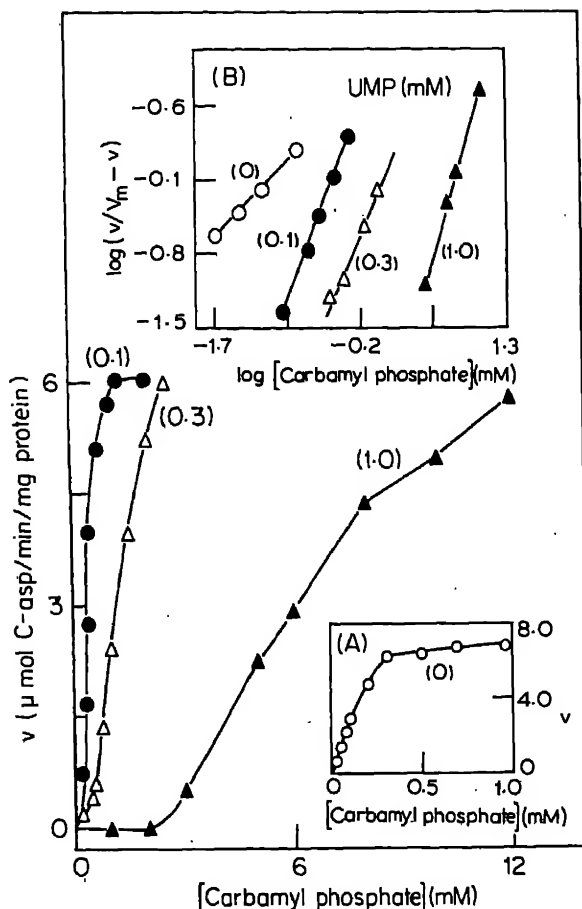
It was interesting to examine whether the enzyme isolated by this method retained its regulatory properties as some regulatory proteins are known to be desensitised during purification (Reddy *et al.*, 1980).

### *Carbamyl phosphate saturation of the enzyme*

The enzyme exhibited a hyperbolic saturation with carbamyl phosphate (figure 2 inset A). The hyperbolic nature of the carbamyl phosphate saturation was confirmed by a Hill plot (figure 2, inset B). When the data was fitted to the Hill equation by a least square analysis, a  $n_H$  value of 1.1 and a  $K_{0.5}$  value of 0.08 mM were obtained. The hyperbolic nature of the saturation was further confirmed by Lineweaver-Burk and an Eadie plots (not shown) both of which were linear. This observation was not in agreement with the results of earlier workers (Rao *et al.*, 1979; Savithri *et al.*, 1978a, b),



**Figure 1.** Molecular properties of mung bean ATCase. **A** and **B**. PAGE was conducted in Tri-glycine buffer, pH 8.6, at 4°C. Bromophenol blue was used as the marker dye. The enzyme (40 µg) was loaded on to 7.5% gels and electrophoresis was conducted using a current of 2.5 mA/tube. The gels were stained for protein using Coomassie brilliant blue R250 and destained with methanol:acetic acid:water (43:7:50). Activity staining was carried out according to a slightly modified procedure of Grayson and Yon (1978). Aspartate transcarbamylase activity was revealed as a white opalescent precipitate of calcium phosphate. **C**. A sepharose 6B column (1 × 50 cm) was equilibrated with 50 mM sodium acetate-acetic acid buffer, pH 4.7 containing 6 M GdmCl (Buffer A) and was calibrated by using ovalbumin (*M*, 45,000), trypsinogen (*M*, 24,000), lysozyme (*M*, 14,300), cytochrome c (*M*, 12,400) and  $\beta$ -lactoglobulin (*M*, 18,400). Blue dextran was used to measure the void volume and methyl green to determine the internal volume of the column. As buffer A was very viscous, the volume of the fractions could not be measured accurately. Hence, the weight of the fractions was measured to calibrate the elution positions of markers and the enzyme. The enzyme (1 mg) or the standard proteins (2 mg each) were denatured in 6 M GdmCl and carboxymethylated at pH 8.0. The denatured protein solution (0.25 ml) was made up to 20% sucrose, 0.2% blue dextran and 0.2% methyl green by adding the solid reagents. Fractions (0.5 g) were collected and the elution weights of protein and markers determined. **D**. A sephacryl S-200 column was equilibrated with 20 mM Tris-acetate buffer, pH 8.0 at 4°C and calibrated using cytochrome c (*M*, 12,400), ovalbumin (*M*, 43,000), conalbumin (*M*, 68,000), yeast hexokinase (*M*, 96,000) and bovine catalase (*M*, 232,000). The enzyme (100 µg) was passed through this column separately and the activity in the fractions is shown in the figure.



**Figure 2.** Saturation of the enzyme with carbamyl phosphate and the effect of UMP on this saturation.

The activity of the enzyme at concentrations of carbamyl phosphate in the range 0 to 1 mM indicated in the inset 2A was determined. The enzyme was preincubated with 0.1 (●), or 0.3 (Δ) or 1.0 (▲) mM UMP for 15 min and the enzyme activity assayed at carbamyl phosphate concentrations indicated in the figure inset 2B Hill plot.

who showed that the enzyme exhibited homotropic co-operative interactions with carbamyl phosphate.

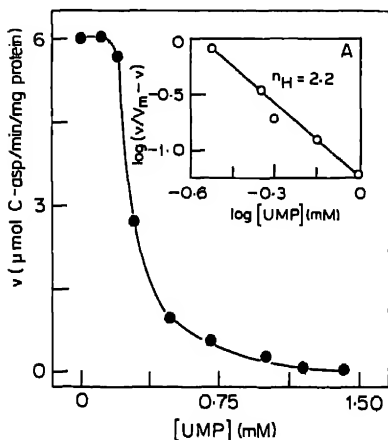
#### *Effect of UMP on carbamyl phosphate saturation*

UMP was earlier (Achar *et al.*, 1974; Savithri *et al.*, 1978a, b) shown to be an allosteric effector of the enzyme. It was therefore of interest to examine the effect of UMP on carbamyl phosphate saturation of the enzyme. The saturation of the enzyme with carbamyl phosphate in the presence of UMP was sigmoidal and the sigmoidicity increased with the increasing concentration of UMP (figure 2). This is more evident in (figure 2, inset B) where the carbamyl phosphate saturations are shown in the form of Hill plots. The

$n_H$  and  $K_{0.5}$  values of carbamyl phosphate saturation increased with increasing concentrations of UMP showing that UMP produced sigmoidicity in the carbamyl phosphate saturation. These results indicate that the purified enzyme retains its heterotropic interaction with UMP.

#### Effect of UMP on the reaction velocity

The inhibition of the enzyme by UMP followed a sigmoid pattern (figure 3) and a Hill plot analysis (figure 3 inset) gave a  $n_H$  value of 2.2 and  $K_{0.5}$  value of 0.26 mM. This result indicated the heterotropic nature of the UMP inhibition and was consistent with the results reported earlier (Savithri *et al.*, 1978b and Rao *et al.*, 1979).



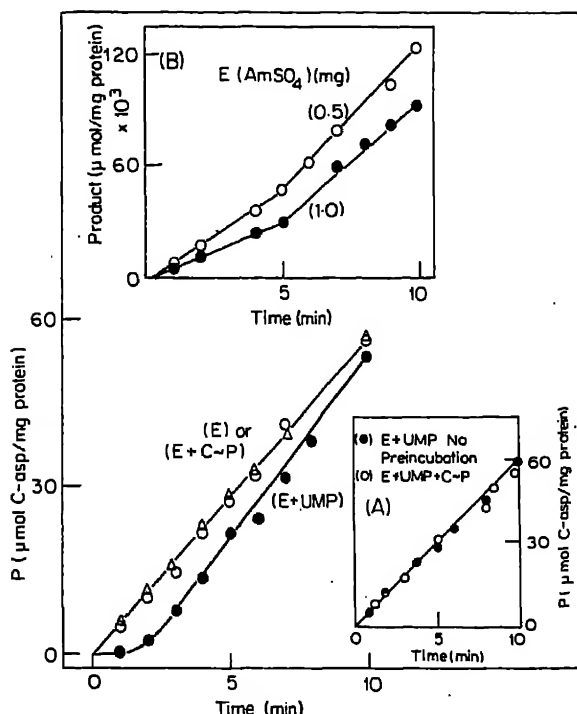
**Figure 3.** The inhibition of the enzyme activity by UMP. The enzyme (1.4  $\mu$ g) was preincubated with concentrations of UMP indicated in the figure and assayed at saturating concentrations of carbamyl phosphate (1 mM) and L-aspartate (10 mM). Inset A. Hill plot.

#### Hysteretic behaviour of the purified enzyme

It was earlier shown that a partially purified enzyme exhibited hysteresis as evidenced by a lag phase in the time course of enzyme catalysed reaction (Rao *et al.*, 1982). It was of interest to examine this property in the purified enzyme.

The progress curve of the enzyme catalysed reaction was linear when the reaction was started by the addition of the enzyme. Similar result was obtained when the enzyme was preincubated with 2.5 mM carbamyl phosphate and the reaction started by the addition of 10 mM L-aspartate. This result indicated the absence of hysteretic behaviour in the purified enzyme and was different from the results reported earlier with a partially purified enzyme preparation (Rao *et al.*, 1982). However, upon preincubating the enzyme with 0.1 mM UMP and starting the reaction with carbamyl phosphate plus L-aspartate a lag phase was observed in the time course of the reaction (figure 4).

When the enzyme was preincubated with 0.1 mM UMP for 30 min followed by a second preincubation with 2.5 mM carbamyl phosphate for 15 min and the reaction started by the addition of 10 mM L-aspartate the progress curve of the reaction did not show any lag phase. No lag phase was observed when the enzyme was not preincubated with UMP (figure 4, inset A). This indicated that the changes produced in the enzyme by UMP leading to a hysteretic response were reversed by carbamyl phosphate. This result

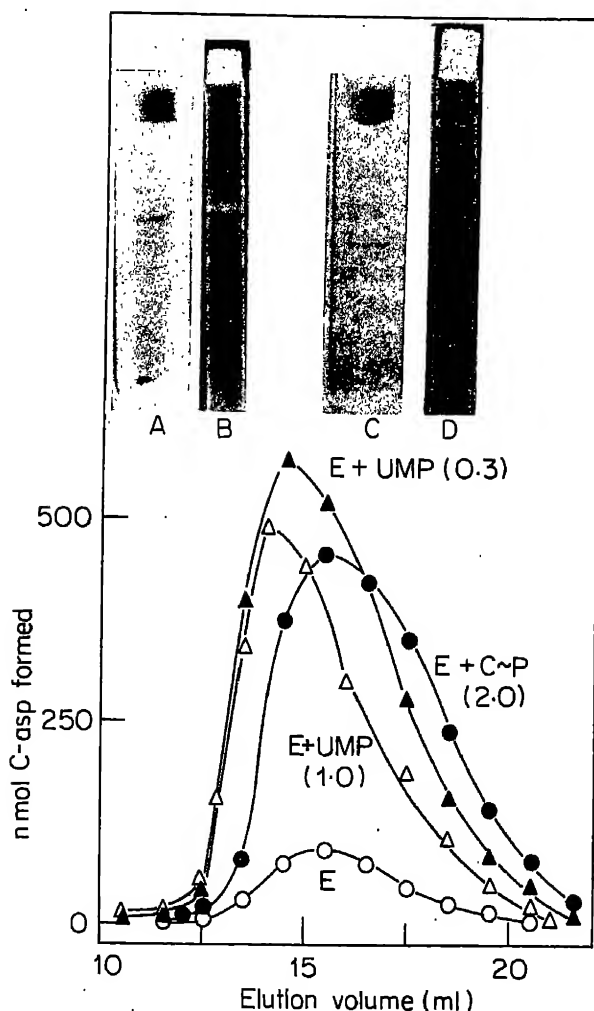


**Figure 4.** Progress of the reaction catalysed by the enzyme. The enzyme ( $12.2 \mu\text{g}$ ,  $\circ$ ) or enzyme preincubated with carbamyl phosphate ( $2.5 \text{ mM}$ ,  $\Delta$ ) for 30 min or UMP ( $0.1 \text{ mM}$ ,  $\bullet$ ) for 30 min in a reaction mixture scaled up to 5 ml and incubated at  $25^\circ\text{C}$ . Aliquots ( $0.5 \text{ ml}$ ) were withdrawn at time intervals indicated and assayed for enzyme activity. *Inset A.* The enzyme was preincubated with  $0.1 \text{ mM}$  UMP for 30 min followed by a second preincubation with carbamyl phosphate ( $2.5 \text{ mM}$ ,  $\bullet$ ) and the reaction was started by L-aspartate ( $10 \text{ mM}$ ). In a control experiment the enzyme was not preincubated with UMP but the reaction mixtures contained  $0.1 \text{ mM}$  UMP ( $\circ$ ). *Inset B.* The partially purified enzyme ( $0.5, 1.0 \text{ mg}$ ) was assayed for activity at different time points in the figure.

is in agreement with those reported for a partially purified enzyme (Rao *et al.*, 1982). This phenomenon was further examined by preincubating the enzyme with  $2 \text{ mM}$  carbamyl phosphate. The enzyme showed two bands on PAGE as revealed by both protein staining (figure 5A) and activity staining (figure 5B). On the other hand in the presence of  $0.3 \text{ mM}$  UMP the enzyme migrated as a single band on PAGE as shown by both protein staining and activity staining (figure 5C and D). These results indicated that the multiple forms of the enzyme observed on PAGE were interconvertible by carbamyl phosphate and UMP. To see whether these different forms were different in their molecular weights, the enzyme was subjected to gel filtration in the presence of these ligands as described below.

#### *Gel filtration behaviour of the enzyme of Sephacryl S-200*

The elution profile of the enzyme on Sephacryl S-200 in the presence of  $2 \text{ mM}$  carbamyl phosphate was similar to that in the absence of any ligands. However, in the presence of



**Figure 5.** Alteration in the molecular weight of the enzyme in the presence of effectors.

A Sephacryl column S-200 ( $1 \times 35$  cm) was equilibrated with 20 mM Tris-acetate buffer, pH 8.0 at 4°C. When the gel filtration was conducted in the presence of ligands the equilibrating buffer contained the appropriate ligands. No ligand (O), UMP (0.3 mM,  $\blacktriangle$ ), UMP (1 mM,  $\triangle$ ); carbamyl phosphate (2 mM,  $\bullet$ ). *Inset A, B, C, D.* PAGE was conducted under conditions described in the legend for figure 1: When the electrophoresis of the enzyme (40  $\mu$ g) was conducted in the presence of carbamyl phosphate or UMP, the electrode tray buffer contained carbamyl phosphate (2 mM) or UMP (0.3 mM). Before electrophoresis the enzyme was preincubated with these ligands for 10 min. A. Protein staining. B. Activity staining for the enzyme preincubated with carbamyl phosphate. C. Protein staining. D. Activity staining for the enzyme preincubated with UMP.

0.3 mM UMP the enzyme was eluted earlier. A greater shift in the elution position was observed when the UMP concentration was increased to 1 mM (figure 5). The results of the gel filtration and electrophoretic behaviour of the enzyme in the presence of ligands



show that the enzyme exists in an equilibrium between different molecular forms and the biospecific ligands can affect this equilibrium.

*Possible consequences of changes in molecular forms on the regulatory properties of the enzyme*

The kinetic properties of an aggregating enzyme depend on the protein concentration of the different molecular forms that have different kinetic properties (Frieden, 1971, 1981). Specific activity of the purified enzyme was examined over a protein concentration range of 2–20  $\mu\text{g}$  per ml. However, there was no change in the specific activity (6.0) of the enzyme over this protein concentration range. It was not possible to increase the enzyme concentration beyond 20  $\mu\text{g}$  per ml as carbamyl phosphate consumption became rapid and was more than 10%. Other experimental difficulties like the non-availability of facilities to measure rapid reactions and the limited availability of the enzyme also precluded the study of the kinetic behaviour of the enzyme at high protein concentration. A slow association–dissociation system is not easily amenable for examination by simple physicochemical methods due to its inherent features. A theoretical paper of Kurganov *et al.* (1976) enables the use of kinetic approach to the examination of these systems even in partially purified preparations. The experimental procedures involve estimation of the specific activity of the enzyme, examination of the substrate and effector saturation profiles at different protein concentrations.

*Kinetic properties of a partially purified enzyme preparation*

The partially purified enzyme (second ammonium sulphate fraction, table 1) showed multiple protein bands on PAGE and activity staining gave multiple bands (data not shown) like the purified enzyme (figures 1A and B). In all the following experiments, precautions were taken to ensure that (a) the product formed was less than 10% of the substrate added by adjusting the reaction times and (b) the initial velocities were being measured.

*Effect of protein concentration on the specific activity of the enzyme*

The specific activity of the partially purified enzyme decreased with increasing protein concentration both at 1 mM and 5 mM concentrations of carbamyl phosphate when the reaction was started by the addition of the enzyme. Preincubation (30 min) with 2 mM carbamyl phosphate resulted in a slight increase in the specific activity of the enzyme as compared with the buffer preincubated control, although a similar decrease in specific activity was observed with increasing protein concentration (data not given).

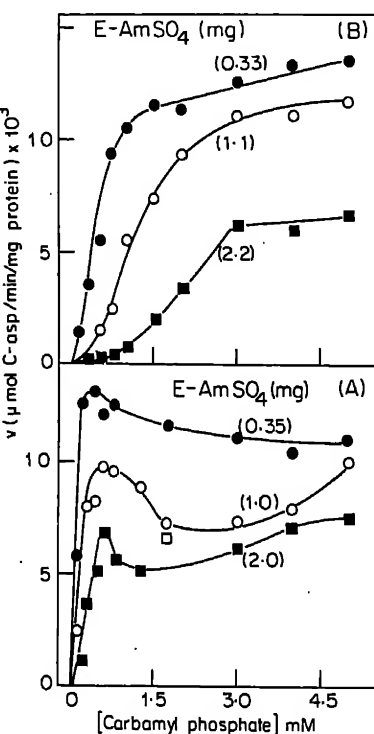
The partially purified enzyme solution (1.5 ml) was made up to 2 mM carbamyl phosphate by the addition of the solid reagent and was set aside for 8 h. It was then dialysed against 1 L of 20 mM Tris-acetate, buffer, pH 8.0 with 4 changes of buffer. When the protein concentration dependence of the specific activity of this preparation was studied, it was observed that over a 10-fold change in protein concentration, there was no change in the specific activity (table 2). The specific activity of this preparation was 0.027 ( $\mu\text{mol}/\text{min}/\text{mg}$  protein) as compared to the buffer treated control which had a specific activity of 0.012.

**Table 2.** Dependence of specific activity on the concentration of a partially purified enzyme preparation treated with carbamyl phosphate.

Protein (mg/ml)	Specific Activity ( $\mu\text{mol}/\text{min}/\text{mg}$ )
0.27	0.026
0.54	0.027
0.82	0.027
1.10	0.026
1.35	0.026
1.63	0.027
2.70	0.026

*The pattern of carbamyl phosphate saturation at different concentrations of the enzyme*

When the reaction was started by the addition of the enzyme, the carbamyl phosphate saturations were complex with a maximum and a plateau region at all the protein concentrations (figure 6A). The velocity of the reaction at each concentration of carbamyl phosphate decreased with increasing protein concentration. On the other hand, when the enzyme was preincubated with various concentrations of carbamyl phosphate and the reaction started by the addition of L-aspartate, classical sigmoid



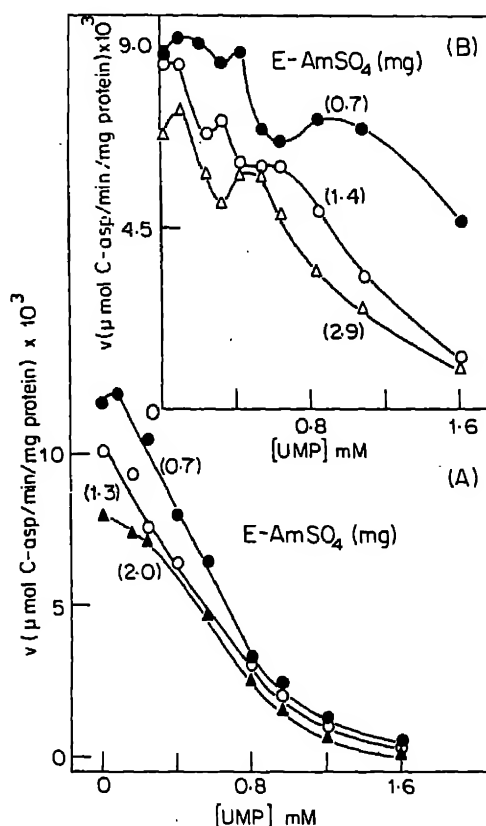
**Figure 6.** A. The saturation pattern of the partially purified enzyme with carbamyl phosphate.

The partially purified enzyme (0.35, ●, 1.0, ○, 2.0, ■) mg was added to reaction mixtures containing different concentrations of carbamyl phosphate indicated in the figure at saturating concentration of L-aspartate. B. The partially purified enzyme (0.33, ●; 1.1, ○; 2.2, ■) mg was preincubated for 30 min at 25°C and added to reaction mixtures containing concentrations of carbamyl phosphate indicated in the figure.

patterns of substrate saturation were obtained (figure 6B). The sigmoidicity increased with increasing protein concentration ( $n_H = 1.5-2.8$ ). The velocity of the reaction at all concentrations of carbamyl phosphate decreased with increasing protein concentration.

*Effect of protein concentration on the UMP inhibition patterns of the partially purified enzyme*

The UMP inhibition pattern of the enzyme at different protein concentrations was sigmoid as indicated by  $n_H$  values greater than 2.0 when the reaction was started by the addition of the enzyme (figure 7A). On the other hand, when the enzyme was preincubated with varying concentrations of UMP and the reaction started by the



**Figure 7.** A. Inhibition of activity at increasing concentrations of UMP at different fixed concentrations of the partially purified enzyme.

The partially purified enzyme (0.7, ●; 1.3, ○; 2.0, ▲) mg was added to the reaction mixtures containing UMP at concentrations indicated in the figure. B. The partially purified enzyme (0.7, ●; 1.4, ○; 2.9, △) mg was preincubated with varying concentrations of UMP indicated in the figure and assayed in the standard reaction mixtures containing the same concentrations of UMP.

addition of carbamyl phosphate plus L-aspartate, the UMP inhibition patterns showed complex behaviour with intermediary plateaus (figure 7B). The velocity of the reaction at all concentrations of UMP decreased with increasing protein concentration.

#### *Time course of the reaction at different protein concentrations*

The partially purified enzyme exhibited hysteresis as indicated by a lag phase followed by a fast phase in the progress curve and this response was dependent on protein concentration (figure 4B).

### Discussion

The enzyme obtained by the method of purification, described in table 1 was extremely stable, probably due to its interaction with carbamyl phosphate. Omission of this ligand in the eluting buffer resulted in a rapid loss of enzyme activity ( $t_{1/2} = 3$  h). Exposure to 2 mM carbamyl phosphate and subsequent dialysis resulted in an enzyme preparation which was stable for months when stored frozen at  $-40^{\circ}\text{C}$ , and this effect was dependent on the ligand concentration. The interaction of the enzyme with AH-Sepharose was probably through a combination of electrostatic and hydrophobic forces (Adler *et al.*, 1975; Shaltiel and Er-El, 1973). The mechanism of stabilization by carbamyl phosphate may not be due to the tight binding of this ligand to the enzyme. This is suggested by the following observations: (i) The enzyme when incubated with L-aspartate in the absence of added carbamyl phosphate did not give rise to the products of the reaction. The amount of enzyme used in this study was sufficient to yield a detectable amount of product; (ii) extensive dialysis had no effect on the stability of the enzyme; (iii) an affinity matrix prepared by coupling the transition state analog, N-phosphonacetyl-L-aspartate (PALA) which has structural features of carbamyl phosphate and aspartate (Collins and Stark, 1971) to AH-Sepharose could also be used for the purification of the enzyme. The enzyme preparation thus obtained was stable even without the addition of carbamyl phosphate and had kinetic properties similar to the carbamyl phosphate-treated enzyme. It could be postulated that the exposure to this compound had stabilized the enzyme in a manner similar to that caused by carbamyl phosphate due to the presence of the structurally similar component in the transition state analogue.

Attempts at dissociating the enzyme into subunits by SDS were unsuccessful. Similar incomplete dissociation by SDS was observed for some hydrophobic proteins (Maddy, 1976; Koistinen, 1980; Graf *et al.*, 1982). The determination of subunit  $M_r$  by gel filtration in 6 M GdmCl indicated a hexameric structure for the mung bean aspartate transcarbamylase. Wheatgerm aspartate transcarbamylase was shown to be a trimer (Yon *et al.*, 1982) which aggregated to higher  $M_r$  forms in the presence of UMP (Grayson and Yon, 1978). The enzyme from *B. subtilis* (Brabson and Switzer, 1975) and the catalytic subunit of *E. coli* ATCase (Weber, 1968) are also trimeric. The enzymes associated with carbamyl phosphate metabolism appear to be mostly trimeric (Vickers, 1981). Thus a trimeric or a multiple of trimeric structure may be a general feature of transcarbamylases.

The purified and stabilized enzyme obtained in this study had properties different

from the earlier preparations (Achar *et al.*, 1974; Rao *et al.*, 1979) in that it did not exhibit homotropic interactions with carbamyl phosphate and hysteresis. However, the enzyme retained the heterotropic interactions with UMP which also induced hysteresis.

The apparent differences between this enzyme preparation and those reported earlier (Achar *et al.*, 1974; Rao *et al.*, 1979) could be reconciled by assuming that treatment with carbamyl phosphate had altered the structure of the enzyme (figure 7B). This explanation finds support in the observation that the ammonium sulphate fraction treated with carbamyl phosphate behaves in an identical manner as that of the purified enzyme.

The multiple activity bands observed on PAGE (figures 1A and B) of the purified enzyme were inter-convertible by carbamyl phosphate (figures 5A and B) and UMP (figures 5C and D) suggesting that they may not be isoenzymes. Similar multiple activity bands on PAGE which were inter-convertible by ligands were observed for some associating-dissociating systems like wheatgerm aspartate transcarbamylase (Grayson and Yon, 1978), bio-synthetic L-threonine dehydratase of *E. coli* (Kagan *et al.*, 1975) and L-threonine deaminase of *Rhodospirillum rubrum* (Feldberg and Datta, 1971). The aggregation of mung bean aspartate transcarbamylase was further confirmed by gel filtration on Sephacryl S-200. UMP shifted the elution profiles of the enzyme towards higher  $M_r$  region. Similar displacement of elution profiles in the presence of allosteric effectors was observed for some aggregating enzymes like isocitrate dehydrogenase (Kelly and Plant, 1981) and homoserine dehydrogenase (Datta *et al.*, 1964).

The complete thermodynamic and kinetic characterisation of an associating-dissociating system requires the use of a number of sophisticated techniques like sedimentation, light scattering, fluorescence polarization etc. (Freiden, 1971). However, the kinetic properties of an associating-dissociating system depends on protein concentration, as an alteration in protein concentration affects the distribution of various  $M_r$  species in equilibrium (Frieden, 1981). Hence, simple kinetic measurements can be used with reasonable success to gain a qualitative understanding of such systems.

The purified enzyme was exposed to carbamyl phosphate which was demonstrated to alter the equilibrium between the different molecular forms of the enzyme. In order to establish that such inter conversions may have a regulatory significance, it was necessary to use a preparation which was not exposed to carbamyl phosphate and hence second ammonium sulphate fraction free from contaminating activities was used.

The partially purified enzyme showed a decrease in specific activity with increasing protein concentration suggesting that the aggregated forms were less active. Since UMP also caused aggregation to higher  $M_r$  forms, the observed inhibition by UMP can be explained on the basis of the lower catalytic activity of the aggregated forms.

The complex substrate and allosteric effector saturation plots with maxima and plateau regions observed in some regulatory enzymes were the subject of two theoretical studies (Tiepel and Koshland, 1969; Kurganov *et al.*, 1976; Kurganov, 1977). Tiepel and Koshland (1969) showed that an enzyme exhibiting mixed co-operativity with negative co-operativity followed by positive co-operativity, gave bumpy saturation curves. Kurganov *et al.* (1976) showed that a hysteretic associating-dissociating enzyme could also produce such complex substrate and allosteric effector saturation

patterns. Since the complex substrate and allosteric effector saturation plots in mung bean aspartate transcarbamylase disappeared depending on the conditions of pre-incubation (figures 7, 8), a hysteric mechanism was more probable than a mechanism involving site-site interactions. Such complex saturation curves were observed for some enzymes like *E. coli* biosynthetic L-threonine dehydratase (Hatfield, 1971; Calhoun *et al.*, 1973; Kagan *et al.*, 1975) and erythrocyte pyruvate kinase (Boivin *et al.*, 1972) which were shown to exhibit a slow association-dissociation of the phenomena (Kagan *et al.*, 1975). The similarities in the properties of mung bean aspartate transcarbamylase and threonine dehydratase permit the postulation of slow association-dissociation phenomenon in the regulation of mung bean enzyme.

### Acknowledgements

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## Tissue esterases of *Exoristina sorbillans* (Uzi fly)

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**Abstract.** Polyacrylamide gel disc electrophoretic technique was used to examine the esterase pattern of head, haemolymph, alimentary canal, ovary and testis of the Uzi fly. The zymograms revealed the varied pattern of esterases both in number and type. This varied pattern suggested several roles for these enzymes present in different tissues.

**Keywords.** Uzi fly; *Exoristina sorbillans*; esterases; tissue distribution; inhibitor specificity.

### Introduction

The Uzi fly, *Exoristina sorbillans*, also known as *Tricholyga bombycis* Beck, is an endoparasite of the mulberry silk worm *Bombyx mori* L. and other strains (Chowdhury, 1970). The infestation has been reported from the states of West Bengal, Karnataka, Tamil Nadu and Andhra Pradesh (Manjeet Jolly, 1981). The Uzi fly causes extensive damage to the silk industry by killing the larvae of the silk worm. Taking this into consideration, some enzymological work was undertaken with special reference to esterases.

Esterases are a group of hydrolytic enzymes occurring in multiple forms with broad substrate specificity. Heterogeneity of esterases from several species of insects has been demonstrated employing electrophoretic techniques and they are shown to be tissue specific (Markert and Hunter, 1969). These enzymes are ubiquitous in nature but their exact physiological significance is not known. Recent studies on esterases from several species implicate them to be involved in the regulation of juvenile hormone titre in the haemolymph (Hammock and Quistad, 1976, 1981), insecticidal resistance (Ahmad and Forgash, 1976; Sudderuddin, 1973), fat mobilization and metabolism (Ahmad, 1976), nerve transmission (Nachmansohn and Wilson, 1951), production of ootheca and vitellogenin (Hooper and Wan, 1969) and lysis of yolk cells in the eggs of silk worm (Kai and Hasegawa, 1973). So far no work has been done on the esterases of the Uzi fly and the present investigation deals with the characterization of the tissue esterases in order to understand their possible role.

### Materials and methods

Maggots were obtained from the Cocoon Market (Ramanagaram) and allowed to pupate. The flies were fed with a 10% glucose solution.

Two to three days old adults were used to collect the tissues. Haemolymph was collected from newly emerged flies by cutting the fore limbs. The whole body, head, testis and ovary were separately homogenised with pre-cooled distilled water, whereas the alimentary canal was homogenised with 1% Triton X-100, and centrifuged at 6700 *g* for 30 min at 4°C. The supernatant was used for electrophoresis.

$\alpha$ -Naphthyl acetate,  $\alpha$ -naphthyl propionate,  $\alpha$ -naphthyl butyrate,  $\beta$ -naphthyl acetate, eserine sulphate, neostigmine bromide, *p*-chloromercuribenzoate and Triton X-100 were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Phosphamidon was a gift from Pesticides and Industrial Chemical Repository (MD-8), Research Triangle Park, North Carolina, USA.

Solutions of inhibitors were prepared in 1% Triton X-100 and dissolved in a small volume of acetone prior to dilution.

### *Electrophoresis*

The electrophoretic system employed was similar to that described by Ornstein (1964) and Davis (1964), using Cyanogum 41. A discontinuous gel system consisting of a 10% separating gel and 4½% spacer gel was used. The electrode buffer used was 0.05 M glycine-sodium hydroxide pH 9.0. An aliquot of the enzyme extract (suitably diluted with 20% sucrose) was carefully layered onto each gel. In the case of alimentary canal, before applying current, Sephadex G-10 (Pharmacia) was layered (about 10–15 mg) onto the gel surface. This method proved most satisfactory with the alimentary canal. A current of 2 mA/gel was applied for 2 h.

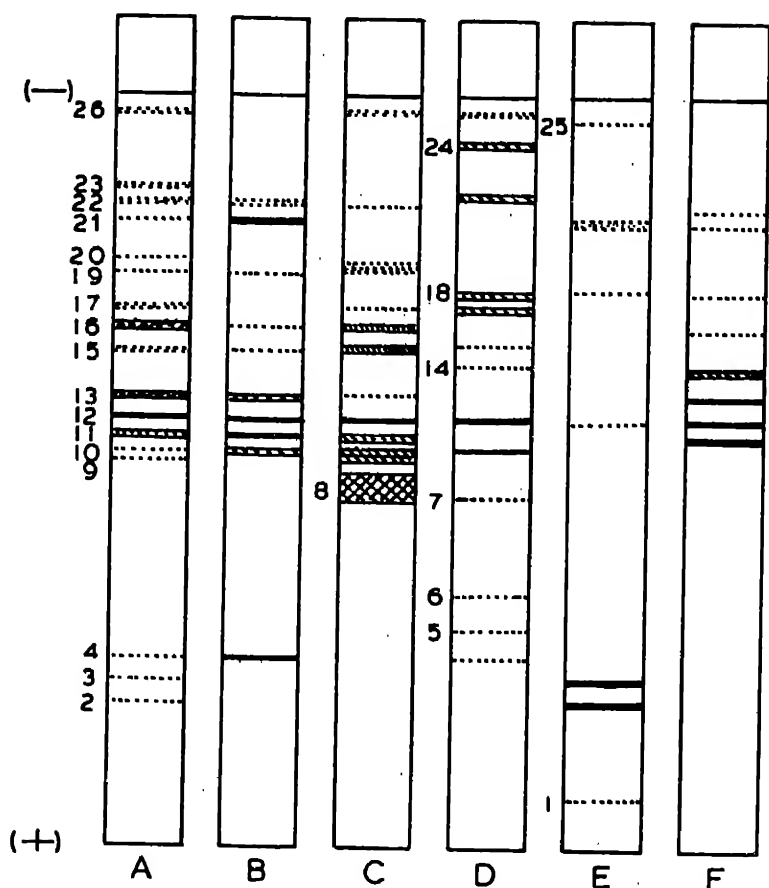
### *Detection of esterase activity*

Esterase activity was detected by placing the gels for 30 min at 37°C in 100 ml of 0.1 M phosphate buffer pH 7.0 containing 40 mg Fast blue RR and 20 mg of the substrate ( $\alpha$ -naphthyl propionate) in 2 ml of acetone. To study the effect of inhibitors, the gels were preincubated in the inhibitor solution for 30 min at 37°C. At the end of the incubation period the gels were stained for esterolytic activity in 0.1 M phosphate buffer pH 7.0 containing the inhibitor, substrate and dye for 30 min at 37°C. A control gel was incubated in 1% Triton X-100 without inhibitor.

The relative mobilities of each esterolytic band was calculated in relation to the mobility of the dye bromophenol blue. The separated bands were indicated by number from the anodic end of the gel.

### **Results and discussion**

The zymograms shown in figure 1 revealed that the tissue esterase patterns vary with respect to type and number. Among the tissues analysed, the maximum number of esterolytic bands (13) was observed in the alimentary canal. On the other hand, only seven bands were noticed in the testis. Tissue specific esterases were observed in haemolymph (band 8), testis (bands 1 and 25) and alimentary canal (bands 5, 6, 7 and 24). Rest of the bands of the tissues investigated were represented in the whole body. Band 12 was prominent in all the tissues except in the testis where it was less intense.



**Figure 1.** Esterase zymograms of Uzi fly using  $\alpha$ -naphthyl propionate as substrate. A, Whole insect; B, head; C, haemolymph; D, alimentary canal; E, testis; F, ovary. Solid bands, very high activity; double hatched, high activity; hatched, moderate activity and dotted bands for low activity.

Other prominent bands observed were in the whole body (bands 13 and 16); head (bands 4, 11 and 21) haemolymph (band 8), alimentary canal (band 10), testis (bands 2 and 13) and ovary (bands 11 and 13).

On the inhibitor specificity, esterases are classified into the following groups (Masters, 1967):

Carboxylesterases (EC 3.1.1.7)–inhibited by carbamates and organophosphates.

Carboxylesterases (EC 3.1.1.1)–inhibited by organophosphates only.

Carboxylesterases (EC 3.1.1.2)–not inhibited by carbamates or organophosphates, but inhibited by PCMB and EDTA.

Carboxylesterases (EC 3.1.1.6)–not inhibited either by carbamates or organophosphates.

The results of the inhibitor studies are shown in figures 2 and 3. These inhibitor studies were carried out in the presence of 1% Triton X-100 to promote the solubilisation of inhibitors. Some of the minor bands shown in figure 1 (1 and 25 of testis, 16 of haemolymph and 23 and 26 of the whole body) did not appear in the presence of 1% Triton X-100 and are not considered for characterization. Treatment with  $10^{-3}$  M phosphamidon (organophosphate) resulted in complete inhibition of bands 9, 10, 11, 12, 17, 19 and 20 in the whole body but these were not affected by  $10^{-3}$  M neostigmine bromide (carbamate). Hence, these esterolytic bands could be classified as carboxylesterases. Using the same criteria, six carboxylesterases each in haemolymph (bands 8, 9, 10, 11, 12 and 13) and alimentary canal (bands 4, 5, 10, 12, 17 and 23), four in head (bands 10, 11, 12 and 13) and ovary (bands 11, 12, 13 and 14) and two in testis (bands 12 and 18) were identified.

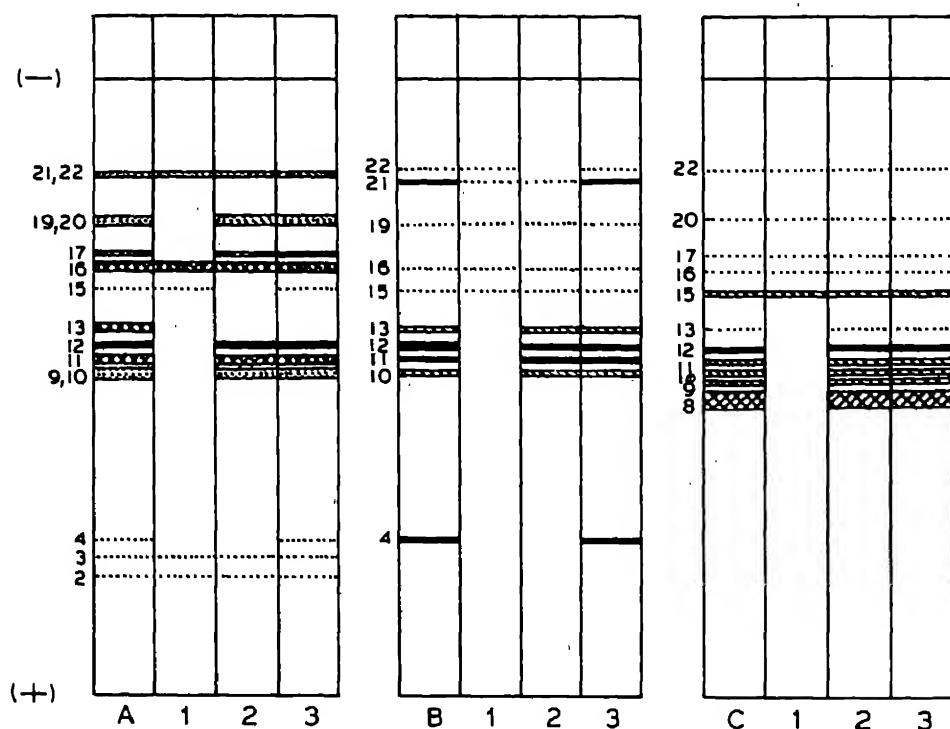


Figure 2. Esterase zymograms of Uzi fly tissues.

A, Whole insect; B, head; C, haemolymph with  $\alpha$ -naphthyl propionate as substrate after incubation with the inhibitors.

1,  $10^{-3}$  M phosphamidon; 2,  $10^{-3}$  M neostigmine bromide;

3,  $10^{-3}$  M *p*-chloromercuribenzoate.

Three bands in the whole body (4, 13 and 15) and the alimentary canal (6, 24 and 26) and two bands in the head (4 and 22) were affected to varying degrees by  $10^{-3}$  M phosphamidon but completely inhibited by  $10^{-3}$  M neostigmine bromide and are

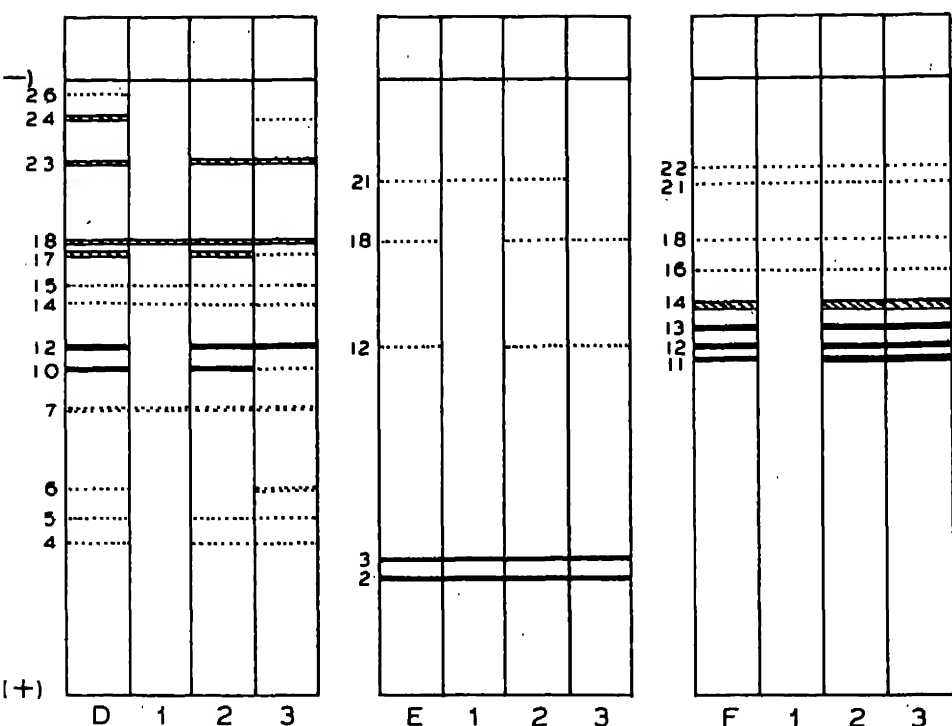


Figure 3. Esterase zymograms of Uzi fly tissues.

D, Alimentary canal; E, testis; F, ovary with  $\alpha$ -naphthyl propionate as substrate after incubation with the inhibitors.

1,  $10^{-3}$  M phosphamidon; 2,  $10^{-3}$  M neostigmine bromide;

3,  $10^{-3}$  M *p*-chloromercuribenzoate.

Solid bands, very high activity; double hatched, high activity; hatched, moderate activity and dotted bands for low activity.

classified as cholinesterases. The ovary, testis and haemolymph were devoid of cholinesterases.

An esterolytic band corresponding to number 21 present in the testis was considered as arylesterase as it was not inhibited by either phosphamidon or neostigmine bromide but inhibited by PCMB.

Two esterolytic bands (2 and 3) in the testis, four each in the head (15, 16, 19 and 21), the alimentary canal (7, 14, 15 and 18), the ovary (16, 18, 21 and 22) and the whole body (2, 3, 16 and 21) and 5 in the haemolymph (15, 16, 17, 20 and 22) were identified as acetylsterases since these were not inhibited by any of the inhibitors used.

Based on the classification using specific inhibitors, carboxylesterases and acetylsterases appeared to be the predominant enzymes in all the tissues examined. Acetylsterases are suggested to play an important role in the intermediary metabolism of nutrients (Slama and Jarolem, 1980). As only one organophosphate was used in the present study, the confirmation of classification as acetylsterase needs further studies.

Although several esterases from different tissues exhibited similar mobilities on the gel, they cannot be considered as identical since they show differential inhibition. For example, the band numbered 17 in the haemolymph is considered as acetylcholinesterase whereas the same band in the whole body and alimentary canal is shown as carboxylesterase. Similarly, the band corresponding to 21 in the testis has been identified as an arylesterase.

The esterases of Uzi fly present in the alimentary canal could only be extracted with 1% Triton X-100 indicating that they may be membrane bound. These are primarily carboxylesterases which perform both physiological and defensive functions. It has been shown in several cases that carboxylesterases in combination with lipases mobilize fats and hydrolyse fatty acid esters in the digestive system (Hipps and Nelson, 1974). Therefore, the presence of six carboxylesterase bands in the alimentary canal of Uzi fly suggests that these enzymes may be involved in digestion of fatty acid esters.

Three esterolytic bands representing cholinesterases are present in the alimentary canal of Uzi fly. These enzymes are also reported to be present in the alimentary canal of the female mosquito *Aedes aegypti* L. (Geering and Freyvogel, 1974) and in the midgut of the gypsy moth *Lymantria dispar* L. (Kapin and Ahmad, 1980). In the case of gypsy moth, it is opined that the presence of cholinesterase may be due to the contamination of gut tissues with peripheral nerve components that are not removed during dissection. At the same time, these enzymes may be involved in acetylcholine metabolism as it is known that choline is required for many metabolic processes. Since cholinesterases constitute a minor proportion compared to carboxylesterases, there may be a possibility that these enzymes are contaminants in the case of Uzi fly.

The Uzi fly head possesses a good complement of cholinesterases, carboxylesterases and acetylcholinesterases. Out of the two cholinesterase bands (4 and 22), the fast moving band (4) is more pronounced when compared with cholinesterase bands present in the alimentary canal (6, 24 and 26).

Several studies suggest that the carboxylesterases of the haemolymph play a role in the regulation of endogenous juvenile hormone (Hammock and Quistad, 1976, 1981). In the haemolymph of the insect *L. decemlineata*, carboxylesterases have been further sub-classified into juvenile hormone-specific esterases and general carboxylesterases (Kramer and De Kort, 1976). Juvenile hormone-specific esterases stain weakly with  $\alpha$ -naphthyl acetate-Fast blue RR, show resistance to diisopropylphosphofluoridate and are completely inhibited by 0.1% Triton X-100. The haemolymph esterases of Uzi fly, on the other hand, hydrolyse  $\alpha$ -naphthyl acetate, are completely inhibited by the organophosphate, phosphamidon, and are resistant to 1% Triton X-100 indicating that these may not represent juvenile hormone-specific esterases.

The reproductive organs are rich sources of esterases. The largest complement of esterases present in the testis of Uzi fly is acetylcholinesterase which is resistant to the high concentration of inhibitors used. Its presence is quite interesting and it is important to understand its role in the reproductive system of the insect. Arylesterase (Aldridge and Reiner, 1972) which is implicated to be responsible for organophosphate hydrolyzing activity, is also shown to be present in the testis of Uzi fly. In *H. Caerulea*, the largest complement of esterases is present in the ovary among the tissues examined (Veerabhadrapa et al., 1978). Carboxylesterase designated as esterase-6 is an enzyme in the reproductive system of *D. melanogaster* and is transferred from the male to the

female during mating (Richmond and Senior, 1981). This enzyme is shown to have an indirect influence on the role of sperm loss in the females. Further, it is suggested that esterase-6 may also be a part of the pheromone system which influences female reproductive behaviour.

It is interesting to note that the Uzi fly ovary contains considerable activity of carboxylesterase. Further studies with carboxylesterases of reproductive system of the Uzi fly on similar lines with those of the fruit fly may help in understanding their role in reproductive behaviour.

### Acknowledgements

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## Involvement of peripheral noradrenaline and 5-hydroxytryptamine in carrageenin-induced pedal oedema in rats\*

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**Abstract.** Role of peripheral and central noradrenaline and 5-hydroxytryptamine in the carrageenin-induced pedal oedema in rats was studied using agents which influence catecholamine synthesis and receptor activity of noradrenaline and 5-hydroxytryptamine. Reserpine, guanethidine,  $\alpha$ -methyl-*p*-tyrosine, diethyldithiocarbamate, 6-hydroxydopamine, phenoxybenzamine, phentolamine, chlorpromazine and yohimbine markedly inhibited carrageenin-induced pedal oedema. However, 6-hydroxydopamine given intracerebroventricularly, 5,6-dihydroxytryptamine, *p*-chlorophenylalanine, lower dose of yohimbine, propranolol, haloperidol, cyproheptadine and mepyramine did not alter the carrageenin-induced oedema, whereas, cyproheptadine and mepyramine given simultaneously, markedly inhibited carrageenin-induced oedema. Our studies indicate that the process of oedema formation in rats by carrageenin involves both the peripheral noradrenaline and 5-hydroxytryptamine.

**Keywords.** Carrageenin-induced oedema; noradrenaline-5-hydroxytryptamine-mediators.

## Introduction

Autacoids are liberated locally in tissues during inflammatory reactions (Ferreira and Vane, 1979). There is sequential release of histamine and bradykinin in the inflammatory process upon subcutaneous injection of carrageenin in rat as shown by testing the exudate (Willis, 1969a,b). Di Rosa *et al.* (1971a,b) showed that histamine and 5-hydroxytryptamine (5-HT) play mediator roles in the carrageenin-induced rat paw oedema. The involvement of central adrenergic and tryptaminergic system in carrageenin-induced pedal oedema in rats has been recently reported (Das *et al.*, 1982). In the present study an approach has been made to find out the involvement of peripheral 5-HT and catecholamines in the mediation of carrageenin-induced pedal oedema in rats.

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Abbreviations used: 5-HT, 5-Hydroxytryptamine; i.c.v., intracerebroventricular; 6-HD, 6-hydroxydopamine; DHT, 5,6-dihydroxytryptamine; RES, Reserpine; MPT,  $\alpha$ -methyl-*p*-tyrosine; DDC, diethyl dithiocarbamate; GUA, guanethidine; PCPA, *p*-chlorophenylalanine; PHENT, phentolamine; CPZ, chlorpromazine; PBZ, phenoxybenzamine; YOH, yohimbine; PROP, propranolol; HALO, haloperidol; CYP, cyproheptadine; MEP, mepyramine; NA, noradrenaline.

## Materials and methods

Wistar albino rats (125–175 g) of either sex obtained from Animal House of the Research Centre, were kept in polycarbonate cages in centrally air-conditioned room at an ambient temperature of  $23 \pm 0.5^\circ\text{C}$  and fed Gold Mohur Hind Lever diet and given water *ad libitum*. The experiments were conducted between 10:00–15:00 h.

Oedema was induced with carrageenin (1% suspension in 0.9% saline) by injecting 0.1 ml under plantar aponeurosis in the right hind paw (Winter *et al.*, 1962). The paw volume, upto the ankle joint, was measured using volume differentiator (Ugo Basile, Italy). The recording was made at hourly intervals for 4 h after carrageenin administration.

Intracerebroventricular (i.c.v.) cannulation of the right lateral ventricle was done under ether anaesthesia and an indwelling cannula was inserted (Feldberg and Lotti, 1967). Rats were used one week after cannulation.

All the drugs were either dissolved in distilled water or suspended in 2% gum acacia except 5,6-dihydroxytryptamine (DHT) and 6-hydroxydopamine (6-HD) which were dissolved in artificial cerebrospinal fluid. The doses and mode of administration of the drugs is summarised in table 1. Drug schedule and doses employed are based on our earlier work (Singh *et al.*, 1978).

Table 1. Dose and mode of administration of drugs

Group <sup>a</sup>	Drug	n	Dose mg/kg <sup>b</sup> (i.p.)	No. of doses	Hours before carrageenin
1.	DHT	4	0.075 i.c.v. <sup>c</sup>	1	72
	6-HD	4	0.25 i.c.v. <sup>c</sup>	1	72
2.	RES	5	2.50 S.C.	1	18
	PCPA	5	100.00	3 × 24 h <sup>d</sup>	24
	DHT	5	75.00	1	72
3.	MPT	5	250.00	1	1
	DDC	5	300.00	1	1
	GUA	5	50.00	1	1
	6-HD	4	250.00	1	72
4.	PBZ	5	10.00	1	1
	PHENT	10	10.00	1	0
	CPZ	5	5.00	1	1
	YOH-0.5	10	0.50 S.C.	1	0
	YOH-2.5	10	2.50 S.C.	1	0
	PROP	5	1.00	2 × 2 h <sup>d</sup>	0
	HALO	5	0.25	2 × 2 h <sup>d</sup>	0
5.	CYP	5	10.00	1	0
	MEP	5	10.00	1	0

<sup>a</sup> All groups consist of 12 control rats except group 1 which consists of 6 rats.

<sup>b</sup> All drugs have been given i.p. unless otherwise mentioned.

<sup>c</sup> Total dose.

<sup>d</sup> Number of dose × h after first dose.

## Results

### Effect of agents influencing catecholamine and 5-HT synthesis on carrageenin-induced pedal oedema

The effect of agents influencing 5-HT and catecholamine synthesis on carrageenin-induced pedal oedema in rats are given in figure 1. Intracerebroventricular administration of both DHT and 6-HD did not affect the carrageenin-induced rat pedal oedema. However, reserpine (RES),  $\alpha$ -methyl-*p*-tyrosine (MPT), diethyl dithiocarbamate (DDC), guanethidine (GUA) and 6-HD significantly ( $P < 0.001$ ) blocked the oedema formation. DHT and *p*-chlorophenylalanine (PCPA) had no effect on pedal oedema induced by carrageenin.

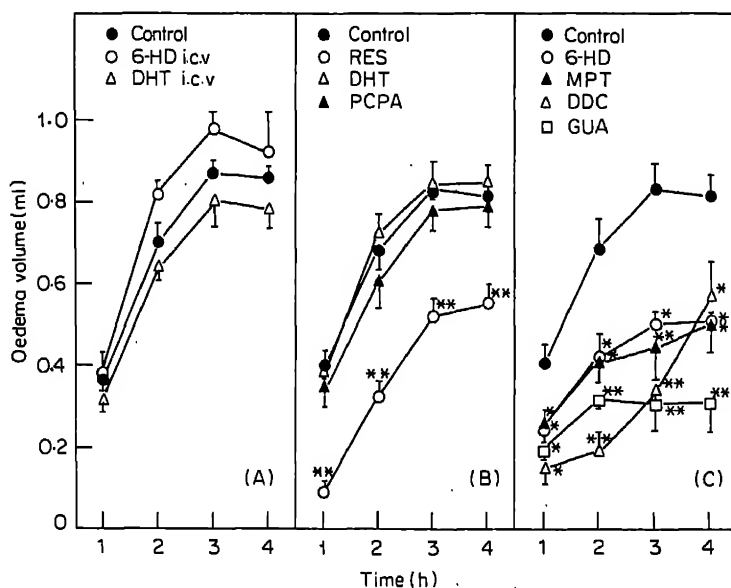


Figure 1. Effect of agents influencing the catecholamine and 5-HT synthesis on carrageenin-induced pedal oedema in rats.

### Effect of receptor blockers on carrageenin-induced pedal oedema

The effect of adrenergic, dopaminergic and serotonergic receptor blockers on carrageenin-induced pedal oedema in rats are given in figure 2. Phentolamine (PHENT), chlorpromazine (CPZ), phenoxybenzamine (PBZ) and yohimbine (YOH) 2.5 mg/kg inhibited significantly ( $P < 0.001$ ) the pedal oedema. However, YOH 0.5 mg/kg, propranolol (PROP) and haloperidol (HALO) had no effect on carrageenin-induced oedema. Although cyproheptadine (CYP) and mepyramine (MEP) *per se* had no effect on carrageenin-induced pedal oedema, but given together, there was significant ( $P < 0.001$ ) inhibition of pedal oedema.

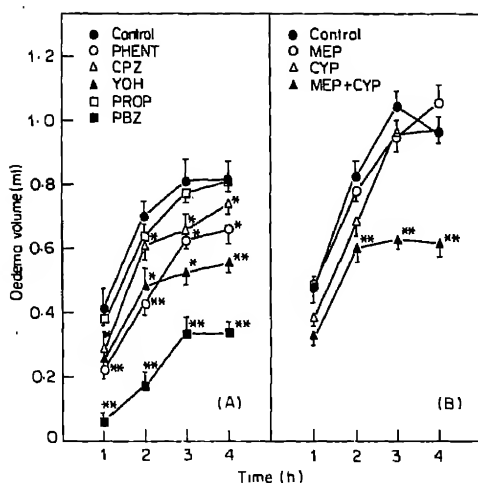


Figure 2. Effect of receptor blockers on carrageenin-induced pedal oedema in rats.

## Discussion

The carrageenin model appears to be unique in acute inflammation in that the role of amines are still poorly understood. In the present study a number of agents which affect the synthesis, storage, release and receptor activity of monoamines, have been used as tools to elucidate the role of catecholamines and 5-HT.

Depletion of both the catecholamines and 5-HT with reserpine resulted in inhibition of the carrageenin-induced pedal oedema, indicating that in the oedema formation either catecholamines or 5-HT or both may be involved.

Inhibition of noradrenaline synthesis by MPT (Spector *et al.*, 1965); DDC (Carlsson, 1966) and peripheral noradrenergic neurone degeneration by 6-HD (Clark *et al.*, 1972) markedly inhibited the pedal oedema, however, central degeneration of noradrenergic neurone by 6-HD (Jacks *et al.*, 1972) did not affect the oedema, indicating the involvement of peripheral noradrenaline (NA) in pedal oedema formation. Inhibition of inflammation by pretreatment with GUA further confirmed peripheral NA mediation of carrageenin-induced oedema.

The postsynaptic  $\alpha$ -adrenoceptor blocking agents *viz.* PBZ, PHENT and CPZ inhibited carrageenin-induced oedema. Presynaptic adrenoceptor blocking agent, yohimbine, in low doses did not affect pedal oedema but in higher doses markedly inhibited pedal oedema. YOH in low doses acts on presynaptic  $\alpha$ -adrenoceptor and in higher doses inhibits postsynaptic  $\alpha$ -adrenoceptors (McCleary and Leander, 1981). PROP, a non-specific  $\beta$ -adrenoceptor blocker and HALO, a dopamine receptor blocker did not affect the carrageenin-induced oedema, thus, suggesting the involvement of postsynaptic  $\alpha$ -adrenoceptors.

To establish the role of 5-HT in carrageenin-induced pedal oedema, PCPA, tryptamine hydroxylase inhibitor, DHT the degenerating agent of central and peripheral tryptaminergic neurone and CYP, 5-HT receptor blocker, were used. All these agents did not inhibit the carrageenin-induced oedema. However, when CYP and MEP were given simultaneously, they inhibited the carrageenin-induced oedema. Di

Rosa *et al.* (1971a) reported that in carrageenin-induced oedema both 5-HT and histamine are released at the same time and in such a concentration that each amine exerts maximal action on vascular permeability. They further observed that the depletion of both of these amines by 48/80 led to a marked reduction in carrageenin-induced oedema. Simultaneous release of these two mediators explains the failure of other workers (Van Arman *et al.*, 1965; Vinegar *et al.*, 1969) to implicate histamine or 5-HT singly in this reaction. Our results are in conformity with the observations of Di Rosa *et al.* (1971a).

In conclusion the results of our present investigation show the involvement of peripheral NA and 5-HT in mediation of carrageenin-induced pedal oedema in rats.

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## Effect of diethylstilbesterol and prolactin on the induction of follicle stimulating hormone receptors in immature and cycling rats

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**Abstract.** Induction of follicle stimulating hormone receptor in the granulosa cells of intact immature rat ovary by diethylstilbesterol, an estrogen, has been studied.

A single injection of 4 mg of diethylstilbesterol produced 72 h later a 3-fold increase in follicle stimulating hormone receptor concentration as monitored by [ $^{125}$ I]-oFSH binding to isolated cells. The newly induced receptors were kinetically indistinguishable from the pre-existing ones, as determined by Lineweaver-Burk plot of the binding data. The induced receptors were functional as evidenced by increased ability of the granulosa cells to incorporate [ $^3$ H]-leucine into cellular proteins.

Neutralization of endogenous follicle stimulating hormone and luteinizing hormone by administering specific antisera had no effect on the ability of diethylstilbesterol to induce follicle stimulating hormone receptors, whereas blockade of endogenous prolactin secretion by ergobromocryptin administration significantly inhibited (~ 30%) the response to diethylstilbesterol; this inhibition could be completely relieved by ovine prolactin treatment. However, ovine prolactin at the dose tried did not by itself enhance follicle stimulating hormone receptor level.

Administration of ergobromocryptin to adult cycling rats at noon of proestrus brought about as measured on diestrus II, (a) a reduction of both follicle stimulating hormone (~ 30%) and luteinizing hormone (~ 45%) receptor concentration in granulosa cells, (b) a drastic reduction in the ovarian tissue estradiol with no change in tissue progesterone and (c) reduction in the ability of isolated granulosa cells to convert testosterone to estradiol in response to follicle stimulating hormone. Ergobromocryptin treatment affected only prolactin and not follicle stimulating hormone or luteinizing hormone surges on the proestrus evening. Treatment of rats with ergobromocryptin at proestrus noon followed by an injection of ovine prolactin (1 mg) at 1700 h of the same day completely reversed the ergobromocryptin induced reduction in ovarian tissue estradiol as well as the aromatase activity of the granulosa cells on diestrus II, thus suggesting a role for proestrus prolactin surge in the follicular maturation process.

**Keywords.** Gonadotropins; receptor induction; prolactin; estrogen; follicle.

### roduction

Over the last several years considerable interest has been generated to study the mechanism of induction of gonadotropin receptors. The receptors for follicle

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Abbreviations used: FSH, Follicle stimulating hormone; LH, luteinizing hormone; DES, diethylstilbesterol; E, ergobromocryptin; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin; Prl, prolactin.



stimulating hormone (FSH) have been demonstrated to be located exclusively in the granulosa cells of the ovary (Midgley, 1973; Zeleznik *et al.*, 1974; Nimrod *et al.*, 1976), but the factor responsible for the induction of FSH receptors in these cells is still not clear. Goldenberg *et al.* (1972) demonstrated increased ovarian uptake of [ $^3\text{H}$ ]-FSH *in vivo* upon diethylstilbesterol (DES) administration. This was due to an increase in the number of granulosa cells with no apparent change in FSH binding sites per cell and the binding association constant (Louvét and Vaitukaitis, 1976). It was concluded (Richards, 1979) that the increased responsiveness of the granulosa cells of hypophysectomized immature rats, pretreated with estrogen, to FSH was not related to changes in the FSH binding sites per cell, but that an estradiol treatment for 12–24 h was facilitatory in FSH inducing its own receptor (Richards *et al.*, 1976).

In the above studies the dose and duration of estrogen treatment was large and prolonged. Further, most studies involved working with hypophysectomized animals. A reinvestigation of some aspects of this problem using intact immature rats and a different dose schedule of estrogen was therefore felt desirable.

In the present study we essentially describe the intrinsic ability of estrogen to induce FSH receptors, the kinetic analysis and functionality of these receptors have also been made. In the course of these studies the involvement of prolactin (Prl) in the inductive process both in estrogen treated immature rat model as well as in the adult cycling rat model has been uncovered.

## Materials and methods

### *Hormones, antisera and chemicals*

Purified ovine follitropin S 1528 C<sub>2</sub>R ( $\sim 50 \times \text{NIH FSH S}_1$ ) was a kind gift from Dr. M. R. Sairam, Canada. Human chorionic gonadotropin (hCG) (CR 123) was obtained from Dr. R. Canfield through NIAMDD, Bethesda, USA. Ovine prolactin (NIH PS 13), ovine FSH (NIH oFSH S15) and radioimmunoassay kits for estimation of rFSH, rLH and rPrl were obtained from NIAMDD, Bethesda, USA. Ergobromocryptin (EBC) was obtained from Sandoz, Basle, Switzerland. The following chemicals were purchased from British Drug House Chemicals, UK and were of AnalaR grade: acetaldehyde, glucose and chloramine-T. Diphenylamine, glacial acetic acid, propylene glycol were obtained from British Drug House Chemicals, Bombay. Estradiol-17 $\beta$  and progesterone were obtained from Steraloids, Inc., Wilton, New Haven, USA. DES,  $\beta$ -mercaptoethylamine hydrochloride, bovine serum albumin were supplied by Sigma Chemical Co., St. Louis, Missouri, USA. Carrier free Na[ $^{125}\text{I}$ ], (2,4,6,7- $^3\text{H}$ ) estradiol-17 $\beta$ , (1,2,6,7- $^3\text{H}$ ) progesterone and L (4,5- $^3\text{H}$ ) leucine were obtained from Radiochemical Centre, Amersham, UK.

Production and characterization of monkey antiserum to oFSH and oLH was carried out as described earlier by Sheela Rani and Moudgal (1977). To determine the amount of rat FSH RP<sub>1</sub> and rat luteinizing hormone (LH)RP<sub>2</sub> (NIAMDD reference preparations) needed to saturate a known volume of antiserum to FSH and LH respectively, a titration using [ $^{125}\text{I}$ ]-labelled rat FSH and LH was performed. Graded doses of rFSH RP<sub>1</sub> were incubated with 0.01  $\mu\text{l}$  of the antiserum to FSH for 12 h followed by incubation with [ $^{125}\text{I}$ ]-rat FSH for 12 h. Sufficient normal monkey serum

and goat antiserum to monkey  $\gamma$ -globulin were added and incubation continued for 2 h, the precipitate obtained was counted for bound radioactivity. All incubations were carried out at 37°C. 5  $\mu$ g of rat FSH RP<sub>1</sub> was found to completely saturate 0.01  $\mu$ l of the antiserum. Using [<sup>125</sup>I]-rat LH and rat LH RP<sub>2</sub> reference preparation, the ability of 0.02  $\mu$ l of the LH antiserum to bind rat LH was determined. 500 ng of rat LH RP<sub>2</sub> (61 times purer than rat LH RP<sub>1</sub>) could completely be neutralized by 0.02  $\mu$ l of the antiserum to LH. Further, the presence of FSH and LH antibody in the serum samples obtained at the time of autopsy from animals treated with 300  $\mu$ l of the FSH and LH antiserum 3 days earlier, was indicated by the ability of 100  $\mu$ l of serum to consistently bind 70 and 60% [<sup>125</sup>I]-iodo rat FSH and LH respectively. Undiluted FSH and LH antisera (100  $\mu$ l) bound 80 and 70% radiolabelled rat FSH and LH respectively. Progesterone antiserum was raised in a rabbit as previously described (Mukku and Moudgal, 1975). Antiserum specific to estradiol-17 $\beta$  was obtained as a kind gift from Dr. P. N. Rao, South West Foundation, Texas, USA. It cross-reacted with estriol to the extent of 0.6%, but showed no cross-reactivity with estrone or C-19 steroids.

#### Animals and treatment

Two month and twenty-one-day old female albino rats of our Institute colony originally derived from the Wistar strain were maintained under a light/dark schedule of 14:10 h. They were fed a standard pellet diet (Hindustan Lever Ltd., Bombay) and given water *ad libitum*. Adult cycling rats were checked for regularity of cycle for at least four consecutive cycles by observing vaginal smears. Those animals which exhibited at least three regular cycles were used in the experiments. DES, progesterone (in 100  $\mu$ l of propylene glycol) and EBC (in 100  $\mu$ l of 50% ethanol) were injected s.c. oFSH (NIH oFSH S15), oPrl (100  $\mu$ l; dissolved in minimal vol. of 0.1 N NaOH and diluted in saline) and monkey antiserum to FSH and LH were injected intraperitoneally.

#### Iodination of hormones

Iodinations of purified oFSH, hCG, rFSH, rLH and rPrl were carried out essentially by the method of Greenwood *et al.* (1963) except that it was done at 4°C, and  $\beta$ -mercaptoethylamine hydrochloride was used as a reducing agent. The specific activities of the radiolabelled oFSH and hCG were in the order of approximately 1–1.25 ( $\times 10^5$ ) and 1.25–1.5 ( $\times 10^5$ ) cpm/ng respectively. Typically 40 to 50% of the iodinated hormone was bound to excess receptor preparation (27000 g pellet obtained from freshly prepared testicular homogenate of 2 months old mature male rats).

#### Antral cell isolation

The animals were sacrificed by cervical dislocation. The ovaries quickly excised, freed of surrounding fat and connective tissues and placed in 2 ml Krebs-Ringer bicarbonate buffer containing 20 mM 4-(2 hydroxyethyl)-1-piperazine ethane sulphonic acid, 0.2% glucose, 0.1% bovine serum albumin (pH 7.2)-Hepes-buffer. All handling of the tissues was done in a petri-dish containing Hepes-buffer and kept on crushed ice. The ovaries from each group of animals were pooled and the total ovarian wet weight was recorded on a torsion balance.

Granulosa cells were expressed into Hepes-buffer as described earlier (Vidyashankar and Moudgal, 1981). The volume of cell suspension of different treatment groups were adjusted on the basis of the ovarian weight so that an approximately equal number of cells per unit volume was obtained. Aliquots of cell suspension (100  $\mu$ l) were used for *in vitro* incubation and DNA estimation. DNA was estimated by Burton's modified diphenylamine method (Giles and Myer, 1965) with the reagent volumes proportionately reduced to yield a 1-ml final volume. Calf thymus DNA was used as standard DNA preparation (sensitivity 5  $\mu$ g/ml).

#### *In vitro incubation and radioimmunoassay*

The granulosa cells (100  $\mu$ l aliquots) were incubated in triplicate with [ $^{125}$ I]-oFSH ( $2 \times 10^5$  cpm/tube) with or without a large excess of unlabelled oFSH in 3 ml glass tubes in a volume of 500  $\mu$ l for 2 h at 37°C in a Dubnoff incubator at 60 oscillations per min. At the end of the incubation the tubes were centrifuged at 1500g at 4°C, the cell pellets were washed with one ml cold Hepes-buffer and counted for bound radioactivity in a Packard Autogamma Counter. LH receptor concentration was monitored using [ $^{125}$ I]-hCG essentially by the method described for FSH receptor. Results are expressed as [ $^{125}$ I]-hormone bound/100  $\mu$ g DNA. This assay has been validated with respect to hormone specificity and saturability. Graded amounts of oFSH (0.1–100 ng/tube) and pregnant mare serum gonadotropin (PMSG) (1 ng–1  $\mu$ g/tube) competed with [ $^{125}$ I]-oFSH for FSH binding sites on the granulosa cells (50  $\mu$ g calf thymus DNA equivalents) obtained from immature rats 72 h after a single injection of 4 mg DES in propylene glycol. oLH and hCG did not compete with [ $^{125}$ I]-oFSH at concentrations upto 5  $\mu$ g/tube. The time course of [ $^{125}$ I]-oFSH binding to granulosa cells showed a biphasic response with an initial rapid phase of binding in the first 15 min followed by a slower phase reaching maximal binding from 45–120 min of incubation. Non specific binding was less than 5 % of the total binding, and was of the order of 1 % of labelled hormone input. [ $^{125}$ I]-oFSH binding to these rat granulosa cells was linearly related to cell concentrations when cell suspensions (in the range of 20–100  $\mu$ g calf thymus DNA equivalents) were incubated with radiolabelled FSH ( $2 \times 10^5$  cpm/tube). Optimal cell concentration and labelled hormone were used in receptor binding studies to obtain saturation of receptors. Typically granulosa cells (50–60  $\mu$ g calf thymus DNA equivalents) in each treatment group were used in radioreceptor assay.

Rat Pri, FSH and LH in the serum were determined by homologous radioimmunoassay using NIAMDD RIA kits. Progesterone and estradiol-17 $\beta$  in the ovarian tissue was estimated after homogenization in buffer and extraction with diethylether (X3) and reconstitution in 0.01 M phosphate buffer, pH 7.4, containing 0.1 % gelatin. The free and bound labelled steroids were separated by dextran coated charcoal treatment. The sensitivity of the assay was 10 and 25 pg, the range 5–500 pg and 10–1000 pg per tube for estradiol and progesterone assays respectively.

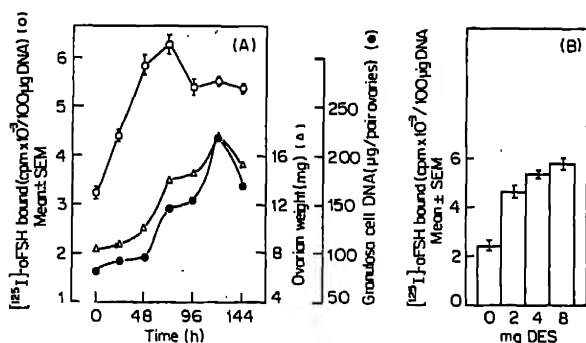
In the following experiments, granulosa cells were obtained from pooled ovaries of rats of each treatment group, wherein the number of rats ranged from 4–8. Each experiment has been repeated more than twice and the results of a typical experiment have been indicated in all the cases.

ults

### Induction of FSH receptors by DES; Time course and dose response

A single injection of 4 mg of DES produced a 3 fold increase in FSH receptor concentration 72 h later (figure 1A). Thereafter a shallow drop in the receptor level was observed which was maintained until 144 h after the hormone injection. The increase in specific activity of FSH receptors ( $[^{125}\text{I}]$ -oFSH bound/100  $\mu\text{g}$  DNA) paralleled the increase in ovarian weight and granulosa cell proliferation as measured by the increases in DNA, suggesting that an induction of FSH receptors had occurred following DES administration.

From the dose response of FSH receptor induction by different doses of DES, it was observed that maximal effect was obtained with 4 mg dose, and consequently in all other studies this dose was used for induction of FSH receptors (figure 1B).



**Figure 1.** Time and dose-response curve illustrating the FSH receptor induction in granulosa cells following a single injection of DES. A. Indicates time course of receptor induction by 4 mg DES.  $P < 0.002$  between 0 h and all other time points. B. Indicates dose response of receptor induction 72 h after injection of different doses of DES.  $P < 0.01$  between 0 and 4 or 8 mg DES. FSH receptor concentration in the isolated cells was monitored as described under Materials and methods. Values are Mean  $\pm$  SEM of triplicate determinations.

### Ability of DES to induce FSH receptors in the presence of gonadotropin antisera

Since estrogens are known to effect pituitary gonadotropin release (Labrie *et al.*, 1978), it was essential to learn whether the FSH receptor induction by estrogens, could be a result of such an effect. The ability of DES to induce FSH receptors remained unchanged even when animals were treated with oFSH and oLH antisera to neutralize endogenous rat FSH and LH (table 1). These antisera in the amounts used were more than adequate to neutralize endogenous rat FSH and LH respectively.

Since in several earlier instances, investigators (Bergink *et al.*, 1973; Palmiter, 1975; Murty and Adiga, 1978) have observed that estrogen is capable of secondary stimulation of specific protein synthesis, it was considered worthwhile to see if a second injection of DES given 96 h after the first one does bring about secondary stimulation of the receptors. No such effect, however, was observed (table 2). Further, progesterone when given at a dose of 2 mg/rat was found not to inhibit the ability of DES to induce FSH receptors (table 3).

**Table 1.** Ability of DES to induce FSH receptors in the presence of gonadotropin antisera.

Treatment	CPM [ $^{125}$ I]-oFSH bound/100 $\mu$ g DNA Mean $\pm$ SEM
Control	2167 $\pm$ 106
DES	4616 $\pm$ 137*
DES + oFSH a/s	5609 $\pm$ 205*
DES + oLH a/s	5548 $\pm$ 46*

Groups of rats received a single injection of 4 mg DES in 0.1 ml propylene glycol or vehicle on day 21. Monkey antiserum (300  $\mu$ l) to oFSH and oLH was injected 2 h prior to DES injection. Controls received 300  $\mu$ l of normal monkey serum.

\*  $P < 0.001$  compared to control value.

**Table 2.** Inability of DES to cause secondary stimulation of FSH receptor induction.

Injection of DES at (h)	Time of autopsy (h)	CPM [ $^{125}$ I]-oFSH bound/100 $\mu$ g DNA Mean $\pm$ SEM
—	96	3125 $\pm$ 150
0	96	7733 $\pm$ 151
0 and 96	168	6425 $\pm$ 159
0	168	2750 $\pm$ 55

Groups of rats received either vehicle or 4 mg DES in 100  $\mu$ l propylene glycol at the times indicated above. The animals were autopsied at the indicated time and FSH receptor concentration in isolated granulosa cells was monitored as described under Materials and methods.

**Table 3.** Effect of progesterone on the ability of DES to induce FSH receptors.

Treatment	CPM [ $^{125}$ I]-oFSH bound/100 $\mu$ g DNA Mean $\pm$ SEM
Control	3250 $\pm$ 90*
Progesterone (2 mg)	3000 $\pm$ 105**
DES (4 mg)	6525 $\pm$ 190*
DES (4 mg) + progesterone (2 mg)	6575 $\pm$ 125**

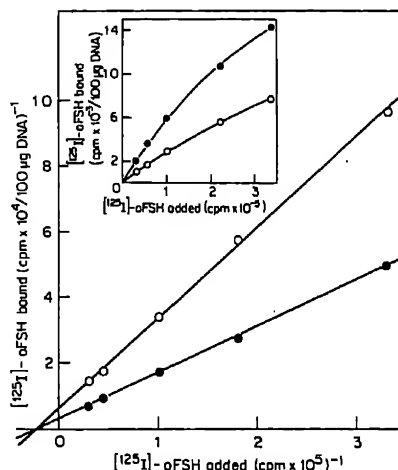
Groups of rats received a single injection of vehicle (control), DES, progesterone, DES + progesterone. The animals were autopsied 72 h after the treatment and FSH receptor concentration in isolated granulosa cells was monitored as described under Materials and methods.

\*  $P < 0.001$ ; \*\*  $P < 0.001$ .

### *Characteristics of the induced receptor*

Lineweaver-Burk plot of binding data obtained by incubating [ $^{125}$ I]-oFSH with granulosa cells from untreated and DES treated rats indicated no change in the affinity of binding of FSH to its receptor although a 2-fold increase in the concentration of the

receptors was observed in the cells from the DES treated group. Thus the newly induced FSH receptors appeared to be kinetically indistinguishable from the pre-existing ones (figure 2).

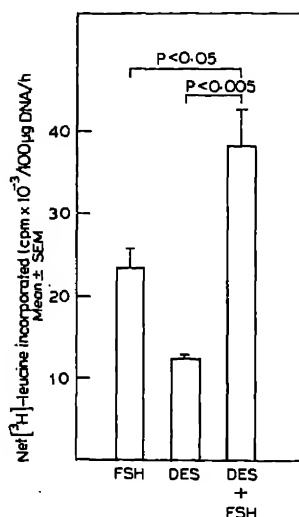


**Figure 2.** Lineweaver-Burk plot of binding data obtained by incubating  $[^{125}\text{I}]$ -oFSH with granulosa cells isolated from ovaries of untreated or 4 mg DES treated rats 72 h earlier. Binding was determined as described under Materials and methods. Linear regression was fitted by the least squares method. Half maximal binding was achieved in both cases at  $4.6 \times 10^5$  cpm. Binding sites (maximal cpm bound/100  $\mu\text{g}$  DNA) calculated from the above, untreated 15,393 (o) and treated 35,983 (•).

The functionality of the induced receptors was assessed by determining the rate of  $[^3\text{H}]$ -leucine incorporation into proteins synthesized *in vitro*, in granulosa cells isolated from ovaries of groups of rats treated 72 h previously with DES or vehicle. While one set of controls and DES treated rats received 20  $\mu\text{g}$  of oFSH (NIH FSH S15), 48 h prior to autopsy, another equivalent set of rats received saline only. It is evident that granulosa cells from DES treated rats respond to FSH significantly (figure 3) showing thereby that the induced receptors are truly functional.

#### Interaction of DES and FSH in inducing gonadotropin receptors in the immature rats

In view of our observation that estrogen induces FSH receptors in the absence of both endogenous LH and FSH and the earlier observations of Richards *et al.* (1976) that FSH induces its own receptors in hypophysectomized rats, the following experiments were undertaken to better understand the interaction between DES and FSH. In the first set, immature rats treated either with or without a single injection of DES, were sacrificed 48 h later either saline or FSH (3  $\mu\text{g}$  of highly purified oFSH, S 1528 C<sub>2</sub>R) given every 12 h, the animals being autopsied 12 h after the last FSH injection. FSH treatment alone increased both FSH and LH receptors in the granulosa cells, whereas DES given alone, increased FSH receptors, but reduced the LH receptors when compared with the untreated controls (table 4). Injection of FSH to DES pretreated immature rats resulted in an apparent further increase in both FSH and LH receptor concentration.



**Figure 3.** Demonstration of the functionality of induced receptors by DES: See text for experimental protocol. Granulosa cells isolated from the pooled ovaries of each group of rats were incubated in triplicate in glass tubes with 10  $\mu$ Ci of (L 4,5-<sup>3</sup>H) leucine (105 Ci/m mol) in a final volume of 500  $\mu$ l of Hepes-buffer, after oxygenation for 20 sec in a shaking incubator for 1 h at 37°C. The time course of [<sup>3</sup>H]-leucine incorporation into cellular proteins was linear with time at 30, 60 and 90 min of incubation. At the end of the incubation, the tubes were spun, the cell pellets washed once with cold Hepes-buffer and resuspended in buffer and an equivalent volume of 20 % trichloroacetic acid was added to precipitate the proteins. The precipitate was washed once with 10 % trichloroacetic acid, dissolved in 0.3 ml of formic acid, transferred to planchets, dried and counted in toluene PPO cocktail. An equivalent volume of cells was used for the estimation of its DNA content. The number of rats in the untreated group was 6 and the DES treated group was 4.

**Table 4.** Interaction of DES and FSH in inducing gonadotropin receptors.

Treatment	CPM [ <sup>125</sup> I]-oFSH bound/ 100 $\mu$ g DNA Mean $\pm$ SEM	CPM [ <sup>125</sup> I]-hCG bound/ 100 $\mu$ g DNA Mean $\pm$ SEM
(A) Control	3546 $\pm$ 141	1265 $\pm$ 4
(B) DES*	5689 $\pm$ 115	518 $\pm$ 140
(C) FSH*	6057 $\pm$ 413	2539 $\pm$ 142
(D) DES + FSH**	8186 $\pm$ 165	3453 $\pm$ 50

Groups of immature rats received the following treatment:

A: 100  $\mu$ l of propylene glycol at 0 h followed by 100  $\mu$ l of saline at 48, 60 and 72 h.

B: 4 mg DES in 100  $\mu$ l propylene glycol at 0 h and 100  $\mu$ l of saline at 48, 60 and 72 h.

C: Same as in group A except 3  $\mu$ g oFSH (S 1528 C<sub>2</sub>R) in saline injected at 48, 60 and 72 h.

D: Same as in group B except 3  $\mu$ g oFSH in saline injected at 48, 60 and 72 h.

All the animals were autopsied at 84 h and the FSH and LH receptor concentration in isolated granulosa cells monitored according to the procedure detailed under Materials and methods.

\*  $P < 0.001$  compared with control.

\*\*  $P < 0.01$  D compared with group B and C.

However, analysis of data showed that this effect of DES and FSH in inducing FSH receptors was an additive effect. FSH was all the same more effective in inducing LH receptors in DES primed rats, when compared with untreated controls suggesting the involvement of estrogen in the LH receptor inductive process by FSH.

In the second set of experiments immature female rats received the first FSH (20 µg NIH oFSH S15) injection along with DES at 0 h, the animals receiving additional FSH injections at 12 h, 24 h and 48 h. The animals were autopsied at 72 h, and the granulosa cells isolated monitored for FSH receptors. The results of this study also showed the effect of DES and FSH on receptor induction to be additive (table 5).

#### *Role of prolactin in the FSH receptor induction by DES*

The well established fact that estrogen brings about short term and long term release of prolactin from the pituitary (Meites *et al.*, 1972; Maurer and Gorski, 1977; Zysek *et al.*, 1981) led us to investigate whether blocking prolactin secretion by EBC had any effect on the DES induction of FSH receptors. EBC (100 µg) inhibited the DES stimulated FSH receptor induction by 30% (table 6). This inhibition was, however, totally relieved with ovine prolactin administration. Prolactin, when tried alone had no effect on the FSH receptor concentration.

**Table 5.** Interaction of DES and FSH in inducing FSH receptors.

Treatment	CPM [ <sup>125</sup> I]-oFSH bound/100 µg DNA Mean ± SEM
Control	7428 ± 216
FSH	11672 ± 695
DES	13498 ± 160
DES + FSH	17780 ± 763

See text, Results for experimental protocol. The granulosa cells were isolated and monitored for FSH receptor concentration as described under Materials and methods.

**Table 6.** Effect of blocking prolactin secretion by EBC on FSH receptor induction by DES.

Treatment	CPM [ <sup>125</sup> I]-oFSH bound/100 µg DNA Mean ± SEM
Control*	2667 ± 238
EBC	2517 ± 555
DES*†	6802 ± 59
DES + EBC**†	5082 ± 213
DES + EBC + oPrI**	7938 ± 60
DES + oPrI	7635 ± 264

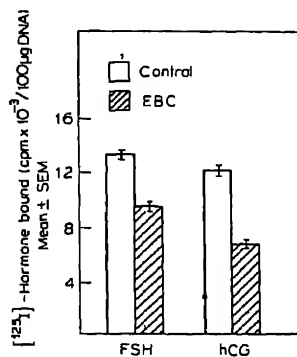
Groups of rats received 4 mg DES or vehicle. 100 µg of EBC was injected 45 min prior to DES injection. oPrI 250 µg was administered every 12 h after the DES injection (total 3 mg/rat). FSH receptor concentration in isolated granulosa cells was monitored 72 h after DES injection as described under Materials and methods.

\*  $P < 0.001$ ; \*\*  $P < 0.001$ ; †  $P < 0.002$ .



### Role of prolactin during cyclical follicular maturation

Administration of EBC to cycling rats at noon of proestrus significantly inhibited FSH ( $\sim 30\%$ ) and LH ( $\sim 45\%$ ) receptor levels (figure 4) in the granulosa cells of the ovaries isolated on diestrus II. This was suggestive of proestrus prolactin surge having a role on the overall follicular maturation process. Administration of EBC on proestrus noon had no effect on the surge of FSH and LH occurring in the evening of the same day but markedly reduced, as expected, Prl levels (table 7). To better understand the role of Prl surge in follicular maturation, rats injected with EBC at noon of proestrus were autopsied on diestrus II and ovarian estradiol-17 $\beta$ , and progesterone levels estimated. Another group of rats received EBC at noon on proestrus followed by ovine Prl at 1700 h the same day to simulate the proestrus Prl peak. Compared to the untreated control a drastic decrease (93 %) in the ovarian tissue estradiol-17 $\beta$  occurred following EBC injection on proestrus. However, the luteal function was not affected by Prl lack as evidenced by no change in the tissue progesterone levels of control and EBC treated rats. Inhibition could be totally relieved by supplementing EBC treated rats with



**Figure 4.** Role of Prl during follicular maturation process in adult cycling rats: Animals ( $n = 3$ ) were given 100  $\mu$ g EBC or vehicle at 1200 h on proestrus and autopsied at 1400 h on diestrus II (3 days later). The granulosa cells obtained from pooled ovaries of each group were monitored for FSH and LH receptor concentration as described under Materials and methods.  $P < 0.01$  and  $P < 0.002$  between control and EBC treated groups for FSH and LH receptor concentrations respectively.

**Table 7.** Effect of administration of EBC on the proestrus FSH, LH and prolactin levels in the serum.

Treatment	ng rFSH RP <sub>1</sub> /ml Mean $\pm$ SEM	ng rLH RP <sub>2</sub> /ml Mean $\pm$ SEM	ng rPrl RP <sub>3</sub> /ml Mean $\pm$ SEM
Control	1070 $\pm$ 162	26.92 $\pm$ 1.68	111 $\pm$ 15*
EBC	816 $\pm$ 16.6	27.2 $\pm$ 4.3	16.6 $\pm$ 1.1*

EBC (100  $\mu$ g in 50% ethanol) or vehicle alone was administered to adult cycling rats on proestrus noon, animals were autopsied at 1700 h of the same day, abdominal vein blood was collected and serum separated. The FSH, LH and prolactin concentration in the serum were estimated by specific radioimmunoassay using NIAMDD RIA kits.  $n$  in each group was 3.

\*  $P < 0.005$ .

exogenous Prl on the evening of proestrus (table 8). The higher tissue progesterone seen in Prl treated group is perhaps a reflection of its luteotropic activity.

In yet another experiment following a protocol described in legend to table 8, granulosa cells isolated from untreated control, EBC and EBC + oPRL treated groups were incubated with testosterone and with or without FSH. The ability of granulosa cells to aromatize testosterone to estradiol in the presence of FSH was reduced in the EBC treated group, supplementing such rats with oPrl once again appeared to restore this activity (table 9).

**Table 8.** Effect of EBC administration at noon of proestrus on the ovarian progesterone and estradiol content measured on diestrus II.

Group	Tissue estradiol pg/100 µg DNA Mean ± SEM	Tissue progesterone ng/100 µg DNA Mean ± SEM
I Control	1373 ± 247*	72.35 ± 11.29
II EBC	59 ± 28*	71.6 ± 9.02
III EBC + oPrl	1259 ± 135	151.63 ± 11.33

The first group of adult cycling rats received 50% ethanol (100 µl) at noon and saline (100 µl) at 1700 h on the day of proestrus. The second group received EBC (100 µl in 100 µl 50% ethanol) at noon of proestrus. The third group received EBC (100 µg in 100 µl 50% ethanol) at noon plus 1 mg of oPrl at 1700 h of proestrus. Animals were autopsied 3 days later, (diestrus II) the ovaries excised and stored frozen in saline until assayed for their contents of estradiol and progesterone. Three animals were used in each group.

\*  $P < 0.005$ .

**Table 9.** Effect of administration of EBC at noon of proestrus on the ability of granulosa cells isolated on diestrus II, to convert testosterone to estradiol-17β.

Treatment	pg estradiol/100 µg DNA		
	No addition	Testosterone $3 \times 10^{-7}$ M	
		-FSH	+FSH
Control	1612 ± 95	5626 ± 315	8820 ± 95*
EBC	971 ± 32	3265 ± 73	2887 ± 150*
EBC + oPrl	1378 ± 61	4859 ± 155	7997 ± 470

The protocol of the experiment is detailed in the legend to table 8. The granulosa cells isolated from pooled ovaries of control, EBC and EBC + oPrl treated groups were incubated for 4 h at 37°C with testosterone and with or without FSH in a total incubation volume of 500 µl. The duration of incubation and the testosterone concentration was chosen according to Vidyashankar and Moudgal (1981). The estradiol secreted into the medium was assayed by an RIA described under Materials and methods. Values are Mean ± SEM of triplicate determinations. Three animals were used in each group.

\*  $P < 0.001$ .

## Discussion

From the foregoing it is evident that DES, an estrogen, is endowed with an intrinsic ability to induce FSH receptors in the immature intact rat. Estrogens have been shown to increase granulosa cell proliferation and growth of a large number of preantral

follicles in the immature hypophysectomized female rat and prolonged treatment with DES is shown to increase ovarian uptake of [ $^3\text{H}$ ]-thymidine and [ $^3\text{H}$ ]-FSH *in vivo* (Goldenberg *et al.*, 1972). In the hypophysectomized immature rats estradiol alone appears to have no effect on the number of FSH receptors when expressed as cpm of [ $^{125}\text{I}$ ]-FSH bound per  $\mu\text{g}$  of DNA of isolated granulosa cells but it does seem to enhance the ability of FSH to increase its own receptors (Louvret and Vaitukaitis, 1976; Ireland and Richards, 1978).

In contrast to the above we find that in the intact immature rat a single injection of DES, in addition to effecting the growth of follicles resulting in granulosa cell proliferation, does bring about a concomitant increase in the specific activity of the FSH receptors expressed here as [ $^{125}\text{I}$ ]-FSH bound per unit DNA. The kinetic property of the induced receptors itself did not change indicating that induction of FSH receptor had truly occurred. Evidence for the functionality of the induced receptors is also provided by demonstrating that granulosa cells from DES treated rats incorporate significantly higher amount of [ $^3\text{H}$ ]-leucine into protein compared to appropriate controls. The observation that even in the absence of endogenous gonadotropins, DES does bring about an increment in receptor concentration, underlines the specificity of the effect. We are presently unable to offer any explanation for this difference in the conclusion of earlier workers and ours excepting to point out that it could stem from the model system and repeated high dosage of estrogen used by them. The results of the experiments wherein both DES and FSH were given together, actually is suggestive of each having an independent inductive effect on granulosa cell FSH receptor levels, administering them together producing an additive effect. This is in contrast to the clear stimulatory effect FSH exhibits with respect to LH receptor induction when given to DES treated rats. Earlier workers have shown that FSH increases the proliferation rate in granulosa cells (Nakono *et al.*, 1975) and more so in combination with estradiol (Rao *et al.*, 1978). Both estrogens and FSH are believed to be essential for the development of LH receptors of rat granulosa cells (Zelevnik *et al.*, 1974; Richards *et al.*, 1976; Nimrod *et al.*, 1977; Sheela Rani *et al.*, 1981). Richards *et al.* (1976) also observed a decrease in LH receptor upon estradiol administration to hypophysectomized immature rats and found an enhanced stimulation of LH receptors in estradiol-hFSH treated rats. We essentially confirm the above results.

The present study has uncovered the possible role of prolactin in inducing/maintaining gonadotropin receptors. Thus the administration of EBC to immature rats prior to DES treatment, significantly blocked the FSH receptor induction by DES, and the inhibition was promptly relieved by prolactin administration. The observation that prolactin alone at the doses tried had no effect in inducing FSH receptor, suggest it having only a permissive role in the FSH receptor inductive process by estrogens.

Extending this line of investigation to cycling rats we have observed that the prolactin surge which appears along with LH and FSH surges on the evening of proestrus could be having an effect on follicular maturation. Blockade of prolactin surge significantly reduced gonadotropin receptors in the granulosa cells isolated from the ovary on diestrus II (*i.e.*, 3 days later). EBC at the dose tried seemed to reduce only the Prl levels, the LH and FSH levels showing no discernable change. This treatment, however, resulted in a drastic reduction in the ovarian tissue estradiol levels on diestrus

II, and that this was a specific effect of Prl lack was shown by the restoration of the estradiol level by exogenous administration of Prl on the evening of proestrus. The ability of granulosa cells isolated from EBC treated rats to aromatize testosterone to estradiol, in response to FSH *in vitro* was also reduced, but this could once again be restored by administering EBC treated rats Prl on the proestrus evening to simulate a Prl surge. These results would thus suggest that prolactin surge could be having a role in the follicular maturation process and perhaps this is being achieved by modulating in some way the effect of FSH on granulosa cells.

Granulosa cells have been shown to possess Prl receptors on their cell surface. The Prl receptors are few in the granulosa cells of immature rat ovary, but as the follicles develop from the preantral to the large antral stage, these receptors apparently increase. Induction of Prl receptors *in vivo* by FSH treatment to estrogenized immature hypophysectomized rats has been demonstrated (Richards and Williams, 1976; Wang *et al.*, 1979). FSH could also stimulate induction of prolactin receptors in cultures of granulosa cells from DES treated immature hypophysectomized rats (Wang *et al.*, 1979). However, Prl has been shown to inhibit FSH stimulated aromatase in cultures of granulosa cells, obtained from DES primed rats (Dorrington and Gore-Langton, 1982). McNatty (1979) has observed that high levels of Prl in the presence of normal plasma FSH levels has antigonadal effects in woman. In the lactating rat, administration of oPrl effectively reduced the increase in nonluteal estradiol following PMSG treatment (Maneckjee *et al.*, 1977). Progesterone synthesis by human granulosa cells *in vitro* is suppressed by high concentrations of Prl (McNatty *et al.*, 1974). Prl has been shown to inhibit basal and gonadotropin stimulated secretion of progesterone and estradiol in human ovaries (Demura *et al.*, 1982). It has been concluded that Prl acts directly on the rat granulosa cells to inhibit the induction of the aromatase activity by FSH, resulting in a decrease in the amount of estrogen synthesized (Dorrington *et al.*, 1983).

The above mentioned observations made in hyperprolactonemic conditions and in *in vitro* cell culture studies are in contrast to the results presented in this study wherein endogenous Prl secretion blocked by EBC reduced receptor concentration while supplementation with exogenous Prl prevented reduction of total ovarian estrogen levels and the aromatase activity, brought about by EBC. Using FSH-primed LH-treated immature rats, Holt and Richards (1975) observed reduced [ $^{125}$ I]-hCG binding to membrane fractions and serum progesterone following ergocryptine administration. Simultaneous treatment with ergocryptine and Prl completely reversed the effects of ergocryptine. Since Prl secretion was blocked with EBC, a drug having a dopaminergic effect, one should view with caution some of the conclusions, as EBC in addition to reducing Prl levels could also have effects at other loci.

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## Effect of hypocholesterolemic agents of plant origin on catecholamine biosynthesis in normal and cholesterol fed rabbits\*

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**Abstract.** The effect of lipid lowering agents of plant origin garlic oil and guggulipid on the levels of catecholamine and dopamine  $\beta$ -hydroxylase activity of normal and cholesterol fed rabbit tissues has been studied. The catecholamine levels and enzyme activity were found to be decreased in cholesterol (500 mg/kg body wt) fed animals. The feeding of garlic oil (5 mg/kg body wt) and guggulipid (100 mg/kg body wt) an exudate of *Commiphora mukul*, to normal rabbits caused significant increase in the dopamine- $\beta$ -hydroxylase activity and catecholamine levels, while the feed helped the hypercholesterolemic rabbits to recover the decrease in catecholamine biosynthesis.

**Keywords.** Catecholamine biosynthesis; dopamine  $\beta$ -hydroxylase; cholesterol; garlic oil; guggulipid; *Commiphora mukul*; hypolipidaemic.

### Introduction

Catecholamines play an important role in atherosclerosis (Nityanand, 1967; Kapoor and Nityanand, 1969; Bhattacharya *et al.*, 1974). Therefore the study of the effect of antiatherosclerotic drugs on catecholamine biosynthesis became important. Berkowitz *et al.* (1973) have reported an increase in norepinephrine (NE) concentration in all tissues and of NE and serotonin (5-HT) in the brain by an antiatherosclerotic agent, pyridinolcarbamate. In recent years, attempts have been made to investigate the lipid lowering activity of plant products for the development of hypolipidaemic drugs. Guggulipid, an exudate from *Commiphora mukul*, has been shown to possess hypolipidaemic activity (Kapoor and Nityanand, 1971, 1976; Nityanand and Kapoor, 1971, 1973, 1975). Garlic oil has also been observed to reduce the lipid levels in serum and tissue of animals (Jain, 1975, 1976). Since catecholamines are known to be involved in hyperlipidaemia and atherosclerosis, the effect of lipid lowering agents of plant origin on the regulation of catecholamine biosynthesis was investigated and its finding is reported here.

\* C.D.R.I. Communication No. 3435.

Abbreviations used: DBH, Dopamine  $\beta$ -hydroxylase; E, epinephrine; NE, norepinephrine; DA, dopamine; 5-HT, serotonin.

## Materials and methods

Male albino rabbits taken from Central Drug Research Institute animal colony and maintained on a standard animal house diet were grouped (6 animals in each group) and fed with cholesterol, garlic oil and guggulipid orally for 3 months as follows:

- Group I: Standard diet (control).
- Group II: Cholesterol (500 mg/kg body wt) in 2.0 ml peanut oil.
- Group III: Garlic oil (5 mg/kg body wt).
- Group IV: Guggulipid (100 mg/kg body wt).
- Group V: Cholesterol (500 mg/kg body wt) in 2.0 ml peanut oil; and garlic oil (5 mg/kg body wt).
- Group VI: Cholesterol (500 mg/kg body wt) in 2.0 ml peanut oil; and guggulipid (100 mg/kg body wt).

At the end of the experimental period the animals were killed by air embolism and brain and heart tissues were quickly removed and chilled in ice.

### *Estimation of lipids*

Serum and tissue cholesterol and triglyceride were estimated by the method of Zlatkis *et al.* (1953) and Van Handel and Zilversmith (1957) respectively. The phospholipids were estimated according to the combined methods of Ames and Dubin (1960) and Chen *et al.* (1956).

### *Catecholamine estimation*

The levels of catecholamines were estimated as reported earlier (Srivastava and Kapoor, 1979b, 1983).

### *Dopamine $\beta$ -hydroxylase assay*

Chilled tissues were homogenized in ice cold 0.32 M sucrose solution. The homogenate was centrifuged at 3500g in Remi cold centrifuge and the supernatant fraction was used for enzyme assay as described earlier (Srivastava and Kapoor, 1979a, 1983). The concentration of the octopamine cleavage product *p*-hydroxybenzaldehyde was determined from the absorbance at 330 nm using a Beckman (model 24) spectrophotometer.

### *Protein estimation*

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

## Results

The levels of serum and tissue lipids were estimated in normal and hypercholesterolemic rabbits (table 1). In the serum of cholesterol-fed animals a 5 times more increase

Table 1. Serum and tissue lipid levels in cholesterol fed rabbits.

Treatment	Total cholesterol	Triglyceride	Phospholipid
<i>Normal</i>			
Serum	82 ± 12.6	50 ± 9.5	155 ± 19.8
Liver	150 ± 18.5	98 ± 10.5	141 ± 18.6
Heart	170 ± 25.5	110 ± 12.0	120 ± 11.7
Aorta	150 ± 22.8	136 ± 17.5	99 ± 10.3
<i>Cholesterol fed</i>			
Serum	550 ± 27.4	265 ± 40.8	556 ± 35.6
Liver	337 ± 40.8	179 ± 18.9	287 ± 21.7
Heart	250 ± 31.9	145 ± 15.5	207 ± 18.5
Aorta	303 ± 41.6	180 ± 16.8	181 ± 14.9

All values are expressed as mg % ± SEM of 6 animals.

$P < 0.01$  as compared with control.

the total cholesterol, triglyceride and phospholipid levels was observed. Tissue lipid levels were also increased 1.5 to 2 fold. The hypolipidaemic agents such as garlic oil and guggulipid were observed to suppress by 22–26% the rise in the lipid levels of serum and tissues of cholesterol fed animals.

Alterations in catecholamine levels in the brain of hypercholesterolemic and hypolipidaemic drug treated rabbits are shown in table 2. Cholesterol feeding caused significant decrease in the catecholamine levels. In normal rabbits, garlic oil increased the epinephrine and norepinephrine levels by more than 60% while rise in dopamine level was about 20% only. Guggulipid increased epinephrine and norepinephrine levels by about 20% and dopamine level by 37%. The fall in the catecholamine levels due to hyperlipidaemia was stemmed by the feeding of lipid lowering agents.

Heart catecholamines were also found to be decreased in cholesterol fed rabbits (table 3). Both garlic oil and guggulipid caused significant rise in catecholamine levels when given to normal rabbit. In hypercholesterolemic rabbits, lipid lowering agents helped recover the decreased levels of catecholamines. Garlic oil feeding to cholesterol

Table 2. Effect of hypolipidaemic agents on catecholamine levels of rabbit brain.

Treatment	Catecholamine level (ng/g wet tissue)		
	Epinephrine	Norepinephrine	Dopamine
Control	63.1 ± 5.4	76.8 ± 4.3	186.5 ± 10.6
Cholesterol	44.0 ± 2.2	46.2 ± 4.1†	139.6 ± 9.3
Garlic oil	102.2 ± 8.6	129.4 ± 8.6	226.6 ± 15.5
Guggulipid	76.3 ± 3.5	90.8 ± 7.2†	255.3 ± 10.7*
Cholesterol + garlic	62.5 ± 2.8	92.5 ± 7.0	199.0 ± 11.3*
Cholesterol + guggulipid	65.7 ± 4.3	88.3 ± 5.9	201.0 ± 13.5†

Values are mean ± SEM of 6 animals.

$P < 0.01$  as compared with control. † $P < 0.05$  as compared with control. \*Not significant.



**Table 3.** Effect of hypolipidaemic agents on catecholamine levels of rabbit heart.

Treatment	Catecholamine level (ng/g wet tissue)		
	Epinephrine	Norepinephrine	Dopamine
Control	265.6 ± 14.3	509.3 ± 21.5	149.0 ± 11.3
Cholesterol	213.5 ± 11.4	349.3 ± 10.9	102.5 ± 9.4 <sup>†</sup>
Garlic oil	376.7 ± 19.3 <sup>†</sup>	555.2 ± 15.1	223.0 ± 14.3
Guggulipid	347.3 ± 12.5	573.3 ± 17.5	170.6 ± 11.5*
Cholesterol + garlic oil	277.6 ± 12.8	526.5 ± 13.5	156.5 ± 7.3
Cholesterol + guggulipid	315.3 ± 10.7	540.5 ± 18.7	145.3 ± 8.9

Values are mean ± SEM of 6 animals.

$P < 0.01$  as compared with controls. <sup>†</sup> $P < 0.05$  as compared with controls. \*Not significant.

fed animals showed an increase of catecholamine levels from normal value, however, norepinephrine rise was more than epinephrine and dopamine. Guggulipid enhanced the epinephrine and norepinephrine levels of hyperlipaemic rabbits with slight reduction in dopamine level as compared to the control group.

The changes in dopamine  $\beta$ -hydroxylase (DBH) activity of rabbit brain and heart on various treatments are represented in table 4. In hypercholesterolemic rabbits, an inhibition of 27% and 40% in the DBH activity of brain and heart respectively was observed. Feeding of garlic oil and guggulipid caused marked increase in the enzyme activity of normal animals. About 2 fold stimulation was found in DBH activity by guggulipid in both the tissues. A complete reversal of the inhibition in DBH activity due to hypercholesterolemia was obtained by feeding of both the lipid lowering agents separately.

**Table 4.** Dopamine  $\beta$ -hydroxylase activity of rabbit tissues.

Treatment	Specific activity*	
	Brain	Heart
Control	45.7 ± 2.3	44.8 ± 3.8
Cholesterol	32.8 ± 1.9	25.7 ± 1.0
Garlic oil	69.3 ± 3.3	77.3 ± 3.7
Guggulipid	89.5 ± 3.8	84.4 ± 4.4
Cholesterol + garlic oil	63.0 ± 4.2	70.0 ± 4.6
Cholesterol + guggulipid	75.4 ± 6.0	76.0 ± 6.9

\*DBH specific activity expressed as nmol octopamine formed per mg protein per 30 min.

Values are mean ± SEM of 6 animals.

$P < 0.01$  as compared with controls. <sup>†</sup> $P < 0.05$  as compared with controls.

## Discussion

Feeding of cholesterol resulted in marked rise of serum and tissue lipid levels showing induction of hyperlipidaemia in rabbits. The antilipaeamic agents of plant origin garlic oil and guggulipid were found to reduce the cholesterol and lipid levels indicating their hypolipidaemic activity. The levels of catecholamines and DBH activity were reduced in hypercholesterolemic rabbits. The decrease in catecholamine levels may be related with decreased biosynthesis by DBH especially in the case of norepinephrine.

The lipid lowering agents, garlic oil and guggulipid enhanced catecholamine levels and DBH activity of rabbit brain and heart. The increase in the levels of catecholamines may be due to their increased biosynthesis by corresponding enzymes. Our results are in close agreement with those reported by Berkowitz *et al.* (1973) for an antilipaeamic agent pyridinolcarbamate showing increase in norepinephrine level of rat heart, brain and mesenteric artery. It was suggested that pyridinol-carbamate may effect the cardiovascular system directly by modifying the norepinephrine content of heart and vascular tissue and possibly indirectly, by raising the concentrations of brain norepinephrine and serotonin.

Garlic oil and guggulipid reversed the decrease in catecholamine biosynthesis due to hypercholesterolemia. A marked increase in norepinephrine levels by these lipid lowering agents in cholesterol fed animals may be correlated with significant stimulation of DBH activity.

It can be concluded that changes in catecholamine levels and DBH activity induced by garlic oil and guggulipid appear to be related with their hypolipidaemic activity and effect on cardiovascular system. Further, the possibility of some direct relation between catechologenic monoamines and atherosclerosis disease is supported by the observations that hyperlipaemic condition alters catecholamine metabolism and disposition.

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## Excretion of pyruvate in nickel toxicity in wild type and $\text{Ni}^{2+}$ resistant mutants of *Neurospora crassa*

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**Abstract.** The parent wild strain *Neurospora crassa* Em 5297a and three  $\text{Ni}^{2+}$  resistant *Neurospora crassa* mutants have been shown to excrete pyruvate into the culture medium in  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  toxicities.  $\text{Ni}^{2+}$  has a more pronounced effect in this regard. The excretion is progressive with growth inhibition and is abolished by  $\text{Mg}^{2+}$  in all strains and by  $\text{Fe}^{3+}$  partially in the Em strain but not in *Neurospora crassa*  $\text{Ni}^{\text{R1}}$ . Pyruvate, citrate and malate supplementation reverse growth inhibition caused by excess  $\text{Ni}^{2+}$ , but with concomitant suppression of  $\text{Ni}^{2+}$  accumulation. It is suggested that one of the features of  $\text{Ni}^{2+}$  toxicity in *Neurospora crassa* is a derangement in carbohydrate metabolism at step(s) beyond pyruvate and that this is possibly due to decreased *in vivo* activity of  $\text{Mg}^{2+}$  dependent processes.

**Keywords.** *Neurospora crassa*; nickel-resistant *N. crassa*; nickel toxicity; pyruvate excretion.

### Introduction

Toxicities of heavy metal ions such as  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Be}^{2+}$  have been extensively studied in *Neurospora crassa* and *Aspergillus niger* (Maruthi Mohan and Sivarama Sastry, 1983; Naidu *et al.*, 1979; Venkateswerlu and Sivarama Sastry, 1972, 1979; Sivarama Sastry *et al.*, 1962a,b; Adiga *et al.*, 1961, 1962) and it is evident that disturbed  $\text{Fe}^{3+}$  or  $\text{Mg}^{2+}$  metabolism is one of the major underlying mechanisms. Such an interaction between essential metal ions such as  $\text{Fe}^{3+}$  or  $\text{Mg}^{2+}$  could affect carbohydrate utilization. Decreased acid production in *A. niger* in metal toxicities is partially reversed by supplemented  $\text{Mg}^{2+}$  (Adiga *et al.*, 1961). In yeast,  $\text{Ni}^{2+}$ , under certain conditions, inhibits anaerobic glucose utilization and this has been suggested to be due to  $\text{Ni}^{2+}$  inhibition of alcohol dehydrogenase (Fuhrmann and Rothstein, 1968). Pyruvate oxidation by cell suspensions of *Aerobacter aerogenes* is inhibited by  $\text{Co}^{2+}$  (Webb, 1970). On the other hand, in *N. crassa*, in  $\text{Be}^{2+}$  toxicity, utilization of glucose is inhibited but not that of pyruvate and it has been postulated that  $\text{Be}^{2+}$  inhibits glycolysis at some step(s) prior to pyruvate (Naidu *et al.*, 1979).

Growth inhibition of *A. niger* due to  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  toxicities can be reversed by added intermediates of carbohydrate metabolism, especially malate and pyruvate

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(Sivarama Sastry *et al.*, 1962a). Pyruvate reverses  $\text{Be}^{2+}$  toxicity without suppression of  $\text{Be}^{2+}$  uptake (Naidu *et al.*, 1979). Enhancement of uptake of metal ions could also be involved; for instance,  $\text{Mg}^{2+}$  uptake is enhanced by glucose, pyruvate, succinate and  $\alpha$ -ketoglutarate in *A. aerogenes* cell suspensions (Webb, 1970). Thus, toxic metal ion interactions with carbohydrate metabolism are complex.

Recently we have isolated three  $\text{Ni}^{2+}$  resistant mutants of *N. crassa* wherein toxic metal ion interaction with essential ions such as  $\text{Fe}^{3+}$  and  $\text{Mg}^{2+}$  is dependent on the nature of the mutant involved (Maruthi Mohan and Sivarama Sastry, 1983). These mutants, *N. crassa*  $\text{Ni}^{\text{R1}}$ ,  $\text{Ni}^{\text{R2}}$  and  $\text{Ni}^{\text{R3}}$  respectively, while being four-fold resistant to  $\text{Ni}^{2+}$ , differ in cross-resistance to other metal ions such as  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$ . In all these mutants,  $\text{Mg}^{2+}$  reverses  $\text{Ni}^{2+}$  toxicity by suppressing the uptake of  $\text{Ni}^{2+}$ . In the case of *N. crassa*  $\text{Ni}^{\text{R3}}$ ,  $\text{Mg}^{2+}$  counteracts  $\text{Co}^{2+}$  toxicity by a similar mechanism. However, in *N. crassa*  $\text{Ni}^{\text{R1}}$  and  $\text{Ni}^{\text{R2}}$ ,  $\text{Mg}^{2+}$  reversal of  $\text{Co}^{2+}$  toxicity does not cause a decrease in  $\text{Co}^{2+}$  uptake. In view of such strain specific differences in toxic ion- $\text{Mg}^{2+}$  interaction and the observation that the pyruvate was excreted into the culture medium as a result of  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  toxicities in these mutants, we have examined some features of pyruvate excretion. The results presented here show that pyruvate excretion due to  $\text{Ni}^{2+}$  toxicity is suppressed by  $\text{Fe}^{3+}$  and  $\text{Mg}^{2+}$ .

## Materials and methods

### Chemicals

Sodium pyruvate,  $\alpha$ -ketoglutaric acid, oxaloacetic acid, L-malic acid, citric acid and  $\text{Fe}^{3+}$ -EDTA were analytical grade products, purchased from British Drug House Chemicals Division, Glaxo laboratories, Bombay. All other chemicals and reagents used were of analytical grade.

### Organisms and growth

*Neurospora crassa* Em 5297a (wild type) and the  $\text{Ni}^{2+}$  resistant strains ( $\text{Ni}^{\text{R1}}$ ,  $\text{Ni}^{\text{R2}}$  and  $\text{Ni}^{\text{R3}}$ ) were grown for 72 h at pH 4.8–5.0, for 72 h in experiments involving preformed mycelia in media as described earlier (Maruthi Mohan and Sivarama Sastry, 1983). In experiments wherein the effects of pyruvate, malate and citrate were to be examined, these solutions, adjusted to pH 4.8–5.0, were separately sterilized and added aseptically to growth media along with  $\text{Ni}^{2+}$ .

### Identification and estimation of $\alpha$ -ketoacids

At the end of the growth period (72 h), the medium was collected quantitatively, and pooled with mycelial washings. When required, this was concentrated to a known volume. Ketoacids herein were identified by paper chromatography after conversion to their 2,4-dinitrophenylhydrazones (Block *et al.*, 1958). Aliquots of the extracts were adsorbed on Dowex-1 (formate), and ketoacids eluted with 6N formic acid, concentrated and chromatographed (Busch *et al.*, 1962). Pyruvate was assayed in the culture medium (Friedmann, 1943).

*Estimation of Ni<sup>2+</sup>*

Mycelia were subjected to wet digestion after thorough washing to free them of adhering Ni<sup>2+</sup> (Sivarama Sastry *et al.*, 1962c) and Ni<sup>2+</sup> content in digests estimated with dimethylglyoxime (Sandell, 1959).

**Results**

Initial experiments with the parent strain (*N. crassa* Em 5297a) and the three Ni<sup>2+</sup> resistant mutants showed significant ketoacid excretion only in the presence of Ni<sup>2+</sup> and Co<sup>2+</sup> at a concentration which caused 50% growth inhibition (I<sub>50</sub>). Chromatographic analyses established that the ketoacid was mostly pyruvate; the only others found were traces of  $\alpha$ -ketoglutarate and oxaloacetate.

Features of pyruvate excretion at I<sub>50</sub> levels of Co<sup>2+</sup> and Ni<sup>2+</sup> are shown in tables 1 and 2. Despite the I<sub>50</sub> values being different from three mutants, they all excreted

Table 1. Pyruvate excretion in Ni<sup>2+</sup> toxicity in *N. crassa*.

Strain	Conc. of Ni <sup>2+</sup> (mM) I <sub>50</sub>	Dry wt. of** mycelia (mg/flask)	Ni <sup>2+</sup> uptake (pg/100 mg dry/wt.)	Pyruvate* (mg/100 mg dry wt.)
Em 5297a	1.0	21	14	2.97
Ni <sup>R1</sup>	1.0	29	39	0.50
	4.0	15	189	4.25
Ni <sup>R2</sup>	4.0	14	329	4.1
Ni <sup>R3</sup>	4.0	14	10	4.8

*N. crassa* strains grown under Ni<sup>2+</sup> toxic conditions (except for the lower Ni<sup>2+</sup> level in the case of Ni<sup>R1</sup>, which was not inhibitory) for 72 h at 30 ± 1°C in 10 ml medium.

\* Values represent pyruvate excreted into the culture medium.

\*\* Weight of control mycelia (No Ni<sup>2+</sup>): Em, 45; Ni<sup>R1</sup>, 30; Ni<sup>R2</sup>, 29; Ni<sup>R3</sup>, 28.

Table 2. Pyruvate excretion in Co<sup>2+</sup> toxicity in *N. crassa*.

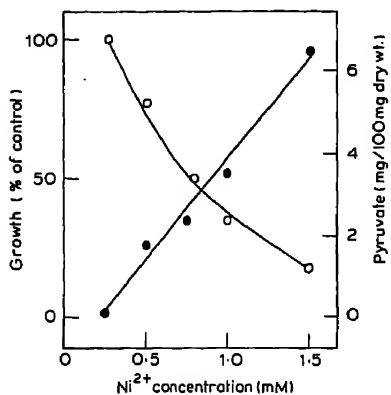
Strain	I <sub>50</sub> Conc. of Co <sup>2+</sup> (mM)	Dry wt. of mycelia* (mg/flask)	Co <sup>2+</sup> uptake (µg/100 mg dry wt.)	Pyruvate** (mg/100 mg dry wt.)
Em 5297a	1.0	23	22	0.8
Ni <sup>R1</sup>	6.0	14	375	1.4
Ni <sup>R2</sup>	6.0	15	481	2.1
Ni <sup>R3</sup>	2.0	13	57	1.8

*N. crassa* strains were grown under Ni<sup>2+</sup> toxic conditions for 72 h at 30 ± 1°C in 10 ml medium.

\* Weight of control mycelia (No Ni<sup>2+</sup>): EM, 45; Ni<sup>R1</sup>, 30; Ni<sup>R2</sup>, 29; Ni<sup>R3</sup>, 28. (controls)

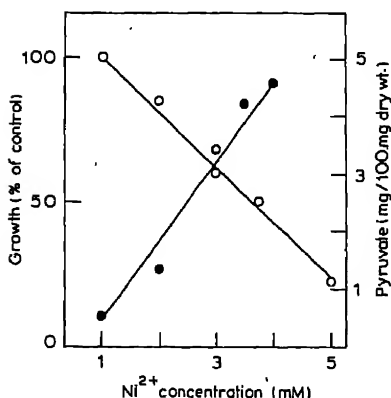
\*\* Values represent pyruvate excreted into the culture medium.

approximately the same amount of pyruvate. However, quantitatively, more pyruvate was excreted in  $\text{Ni}^{2+}$  toxicity. It may be noted that at 1 mM  $\text{Ni}^{2+}$  concentration, which was not toxic to *N. crassa*  $\text{Ni}^{\text{R1}}$ , the parent strain Em excreted more pyruvate than the mutant. Consequently, further studies were conducted on  $\text{Ni}^{2+}$  toxicity in *N. crassa*  $\text{Ni}^{\text{R1}}$  (as a typical  $\text{Ni}^{2+}$  resistant strain). Results presented in figures 1 and 2 show that the pyruvate excretion was a function of growth inhibition with increasing  $\text{Ni}^{2+}$  concentration in both the strains.



**Figure 1.** Influence of  $\text{Ni}^{2+}$  toxicity on growth and pyruvate excretion in *N. crassa* Em 5297a. *N. crassa* Em was grown with different levels of  $\text{Ni}^{2+}$  for 72 h at  $30 \pm 1^\circ\text{C}$ . Media and mycelial washings were pooled and the pyruvate content estimated.

For experimental details see text. (O), Growth; (●) pyruvate.



**Figure 2.** Influence of  $\text{Ni}^{2+}$  toxicity on growth and pyruvate excretion in *N. crassa*  $\text{Ni}^{\text{R1}}$ . *N. crassa*  $\text{Ni}^{\text{R1}}$  was grown with different levels of  $\text{Ni}^{2+}$  for 72 h at  $30 \pm 1^\circ\text{C}$ .

Experimental details as in figure 1. (O), Growth; (●) pyruvate.

Earlier studies had shown that  $\text{Ni}^{2+}$  toxicity in the Em strain was fully counteracted by  $\text{Fe}^{3+}$  and  $\text{Mg}^{2+}$  ions and the reversal of inhibition by  $\text{Mg}^{2+}$ , but not by  $\text{Fe}^{3+}$ , was associated with suppression of  $\text{Ni}^{2+}$  uptake (Sivarama Sastry *et al.*, 1962b). The effects

of these two ions on pyruvate excretion were therefore studied under conditions of reversal of  $\text{Ni}^{2+}$  toxicity. Pyruvate excretion was fully inhibited by 3 mM  $\text{Mg}^{2+}$ . On the other hand, 2 mM  $\text{Fe}^{3+}$ -EDTA complex ( $\text{Fe}^{3+}$ -EDTA complex was found to be a better source of iron for *N. crassa*, Venkateswerlu and Sivarama Sastry, 1973) reversed growth inhibition fully, but reduced pyruvate excretion only by about 50%. In a typical experiment, on a cell dry weight basis, pyruvate accumulated in the presence of  $\text{Ni}^{2+}$  (mg/100 mg dry wt.) was 2.97 as compared to 1.53 in presence of  $\text{Ni}^{2+}$  and 2 mM  $\text{Fe}^{3+}$ -EDTA. Likewise in *N. crassa*  $\text{Ni}^{\text{R1}}$ ,  $\text{Mg}^{2+}$  at 8 mM concentration prevented pyruvate excretion due to 4 mM  $\text{Ni}^{2+}$ ;  $\text{Fe}^{3+}$  was not tested herein, since it does not counteract  $\text{Ni}^{2+}$  toxicity in *N. crassa*  $\text{Ni}^{\text{R1}}$  (loc. cit).

The influence of added pyruvate, citrate and malate on  $\text{Ni}^{2+}$  toxicity in Em and  $\text{Ni}^{\text{R1}}$  strains was also examined. The results (table 3) show that all of them, at concentrations which reversed growth inhibition, suppressed  $\text{Ni}^{2+}$  uptake.

**Table 3.** Effects of pyruvate, citrate and malate on  $\text{Ni}^{2+}$  toxicity in *N. crassa*.

Strain	Organic* acid	Dry wt. (mg)	$\text{Ni}^{2+}$ uptake ( $\mu\text{g}/100$ mg dry wt.)
Em 5297a	—	12	14
	Pyruvate	41	4.9
	Citrate	40	5.0
	Malate	42	7.3
	—	14	189
$\text{Ni}^{\text{R1}}$	Pyruvate	32.5	73.7
	Citrate	28.5	15.9
	Malate	30	23.2

\* Organic acids were supplemented at 100 mg/10 ml medium. Toxic concentration of  $\text{Ni}^{2+}$  used: Em, 1.0 mM;  $\text{Ni}^{\text{R1}}$ , 4.0 mM. Growth for 72 h at  $30 \pm 1^\circ\text{C}$ , pH 5.0. Control weights of mycelia (with no  $\text{Ni}^{2+}$  in medium) were; Em, 50 mg;  $\text{Ni}^{\text{R1}}$ , 31 mg.

## Discussion

In *N. crassa* (Sivarama Sastry *et al.*, 1962b), *A. niger* (Adiga *et al.*, 1961) and in several bacteria (Abelson and Aldous, 1950) there is good evidence that some of the effects of heavy metal toxicities are due to an induced deficiency of  $\text{Mg}^{2+}$  caused by excessive intracellular toxic ion concentrations. The present study suggests that the excretion of pyruvate in  $\text{Ni}^{2+}$  toxicity in *N. crassa* is probably due to deranged  $\text{Mg}^{2+}$  metabolism. In the case of added  $\text{Fe}^{3+}$ , growth inhibition due to  $\text{Ni}^{2+}$  is fully counteracted but there is only a partial suppression of pyruvate excretion.

In *N. crassa*,  $\text{Fe}^{3+}$  counteracts  $\text{Ni}^{2+}$  toxicity by apparently translocating intracellular  $\text{Ni}^{2+}$  from sites wherein  $\text{Ni}^{2+}$  is toxic to others at which  $\text{Ni}^{2+}$  binding is not inhibitory (Sivarama Sastry *et al.*, 1962b). Consequently, the limited ability of  $\text{Fe}^{3+}$  to suppress pyruvate excretion even when it restores the growth rate to normal suggests that the intracellular sites at which  $\text{Ni}^{2+}$  is inhibitory to carbohydrate metabolism are



those which depend on  $Mg^{2+}$ . The reversal of  $Ni^{2+}$  toxicity by added intermediates such as pyruvate, citrate and malate is not a metabolic effect but apparently related to limiting  $Ni^{2+}$  uptake.

It has been suggested earlier that in *A. niger*, the primary metabolic lesions in carbohydrate metabolism due to metal toxicities are at steps involved in the formation of pyruvate and malate (Sivarama Sastry *et al.*, 1962a).  $Be^{2+}$  toxicity inhibits glycolysis at some step(s) prior to pyruvate (Naidu *et al.*, 1979). The present data indicate that  $Ni^{2+}$  (and  $Co^{2+}$ ) toxicity in *N. crassa* affect not the biosynthesis of pyruvate but rather its subsequent metabolism. Thus, the site(s) of action of different metal ions on carbohydrate metabolism depend, apparently, both on the metal ion in question as well on the organism.

### Acknowledgement

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## Purification of bovine and porcine enterokinase by affinity chromatography with immobilized kidney bean enterokinase inhibitor

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**Abstract.** A specific enterokinase inhibitor isolated from kidney bean (*Phaseolus vulgaris*) was immobilized on Affigel-10. Solubilized preparation of bovine and porcine enterokinases were bound to this matrix at pH 7.5 and the complex was dissociated by elution with 10 mM HCl, resulting in the isolation of the enzymes in homogeneous form as judged by gel chromatography on Sephadex G-200, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. However, human enterokinase could not be purified by this method in sufficient yield since it did not bind strongly to the insolubilized inhibitor.

**Keywords.** Affinity chromatography; enterokinase isolation; bovine and porcine; kidney bean enterokinase inhibitor.

### Introduction

Enterokinase (EC 3.4.21.9) a membrane bound serine protease, present in the duodenum, specifically and rapidly converts trypsinogen to trypsin thus triggering the conversion of other zymogens to active enzymes. Purification of this enzyme using non-specific immobilized inhibitors has been reported (Anderson *et al.*, 1977; Liepnicks and Light, 1979). In a recent communication from this laboratory (Jacob *et al.*, 1983), the isolation and characterization of a specific enterokinase inhibitor from kidney bean (*Phaseolus vulgaris*) were described. In this paper, we report the studies on the isolation of enterokinases from different species using immobilized kidney bean inhibitor.

### Materials and methods

Crude porcine enterokinase, Dalton mark VI, cross-linked hemocyanin, Trizma (Tris-hydroxymethyl aminomethane) base, 2-mercaptoethanol and  $\alpha$ -N-benzoyl DL-arginine *p*-nitroanilide (BAPNA) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Sephadex G-200 was got from Pharmacia Fine Chemicals, Uppsala, Sweden. Affigel-10 (cross-linked agarose with N-hydroxysuccinimide as functional group) was the product of Bio-Rad Laboratories, La Jolla, California, USA.

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Abbreviations used: BAPNA,  $\alpha$ -N-Benzoyl DL-arginine *p*-nitroanilide;  $M_r$ , molecular weight; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

Bovine trypsinogen free of trypsin was prepared as described by Willimowska-Pelc and Mejbaum-Katzenellenbogen (1978). Other chemicals were of analytical grade.

All operations were carried out at 4°C unless mentioned otherwise. Bovine duodenum was collected from a slaughter house and human duodenum was obtained during autopsy at the Kasturba Medical College. The mucosal cells were collected by gentle scraping with a spatula and stored at -4°C. The mucosa was extracted with three volumes of 0.05 M Tris-HCl buffer pH 8.0 containing 2% deoxycholate by continuous stirring for 2 h. The homogenate was centrifuged at 10000 *g* for 20 min. The supernatant was acidified to pH 6.0 with 1 N acetic acid to precipitate deoxycholate. The suspension was centrifuged at 10000 *g* for 20 min. The supernatant was adjusted to pH 8.0 with 1 N NaOH and subjected to ammonium sulphate fractionation. The precipitate formed during the increase in the concentration of the salt (from 40% to 70% saturation) was collected by centrifugation at 10000 *g* for 30 min, dissolved in 0.005 M Tris-HCl pH 7.5 containing 0.02 M NaCl and dialyzed overnight against the same system. The dialyzed fraction was used for further purification.

Enterokinase inhibitor from kidney bean was prepared as described earlier (Jacob *et al.*, 1983). The inhibitor (20 mg protein) was dissolved in 5 ml of 0.02 M sodium phosphate buffer pH 7.0 and dialyzed against 100 volumes of the same buffer for 8 h. Affigel-10 (wet volume 10 ml) was washed with 3 volumes of isopropanol and then with one volume of 0.02 M phosphate buffer pH 7.0. The gel was allowed to react with the enterokinase inhibitor for 48 h with occasional shaking. The unreacted groups of Affigel-10 were blocked by treating with 1.0 ml of 1 M ethanolamine (pH 8.0) for 2 h. The immobilized inhibitor was washed with 200 ml of 0.02 M phosphate buffer pH 7.0 followed by 200 ml of 0.01 M HCl. The matrix was stored suspended in 0.01 M HCl. Before chromatography the matrix was packed into a column and washed with 0.01 M Tris-HCl buffer pH 7.5 containing 0.1 M NaCl until the effluent was free of ultra-violet (280 nm) absorbing materials.

Enterokinase was assayed by activation of bovine trypsinogen to trypsin at pH 5.0 and then measuring the activity of trypsin at pH 8.0 with BAPNA as substrate (Erlanger *et al.*, 1961). The activation system contained 40  $\mu$ mol of Tris-acetate pH 5.0, 1  $\mu$ mol of CaCl<sub>2</sub>, 100  $\mu$ g of bovine trypsinogen and enterokinase solution in a volume of 1.0 ml. After 10 min incubation at 37°C, 2.0 ml of 2 mM BAPNA in 0.05 M Tris-HCl pH 8.0 was added and the solution was incubated for 15 min at 37°C. The reaction was stopped by the addition of 1.0 ml of 30% acetic acid and the colour was read at 410 nm. One unit of enterokinase is defined as the amount that liberated 1.0  $\mu$ mol of *p*-nitro-aniline per min under the assay conditions.

For the purification of enterokinase by affinity chromatography commercial porcine enterokinase solution or the ammonium sulphate fraction of bovine or human duodenum was allowed to flow through the inhibitor: Affigel-10 column (1.5  $\times$  5.7 cm, bed volume 10 ml) at a flow rate of 10 ml/h. The column was washed with 200 ml of the equilibration buffer (0.01 M Tris-HCl pH 7.5 containing 0.1 M NaCl) and then eluted with 0.01 M HCl. Ten ml fractions were collected. The acidic eluant was collected in tubes containing 2 ml of 0.2 M Tris-HCl buffer pH 8.0 so that the final pH was 7.5. This prevented the inactivation of enterokinase.

Polyacrylamide gel electrophoresis (PAGE) was performed on 7% gel according to the method of Davis (1964) in 0.095 M Tris-glycine buffer pH 8.3 with a current of 3 mA per

tube. The protein concentration used was in the range of 40–80  $\mu$ g. The molecular weight ( $M_r$ ) of the purified enzyme was determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (0.1 % SDS, 5 % gel) as described by Weber *et al.* (1972). The run was for 10 h with a current of 7 mA/tube. The protein bands were stained with 0.25 % Coomassie Brilliant Blue R-250 in methanol:acetic acid:water (5:1:5). As marker proteins cross-linked hemocyanin, bovine serum albumin, ovalbumin, pepsin,  $\beta$ -lactoglobulin, trypsinogen and lysozyme were used.

The purified enterokinase was subjected to chromatography on Sephadex G-200. One ml of purified bovine enterokinase (600  $\mu$ g protein, 51 units) or porcine enterokinase (1060  $\mu$ g protein, 44 units) solution was loaded onto a column of Sephadex G-200 (0.9  $\times$  63 cm, bed volume 40 ml) equilibrated with 0.01 M Tris-HCl buffer pH 7.5 containing 0.1 M NaCl and eluted with the same buffer. One ml fractions were collected and the flow rate was 6 ml/h. Fractions were assayed for protein and enterokinase.

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

## Results

Enterokinase inhibitor from kidney bean was immobilized on Affigel-10 and this preparation was found to be active and stable when stored at 5°C in 10 mM HCl at least for 2 months. This preparation could be successfully used for a one-step purification of solubilized bovine and porcine enterokinases. The regenerated column can be reused at least 10 times without marked effect on enzyme recovery.

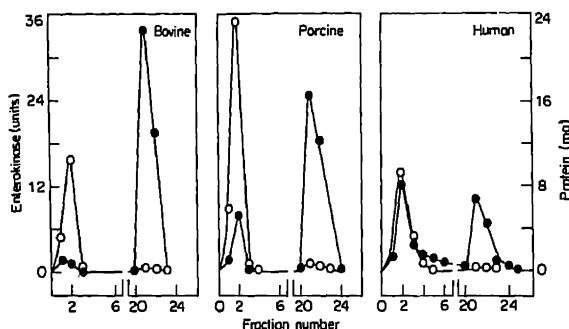
In table 1, the magnitudes of retention of different enterokinases on the immobilized ligand are shown. It was observed that a small proportion of bovine enterokinase was not retained on the column and this proportion increased with increase in the load of enzyme. With porcine enterokinase also a similar phenomenon was observed, but the proportion of enzyme not retained was more in this case. The cause of elution of part of the enzyme in the washings does not appear to be a overload of the column. In the case of the human enterokinase nearly 50 % of the enzyme (recovered activity) was eluted in the washing itself even with a small load. Typical elution profiles of the three

Table 1. Behaviour of different enterokinases during affinity chromatography on enterokinase inhibitor-Affigel-10 column.

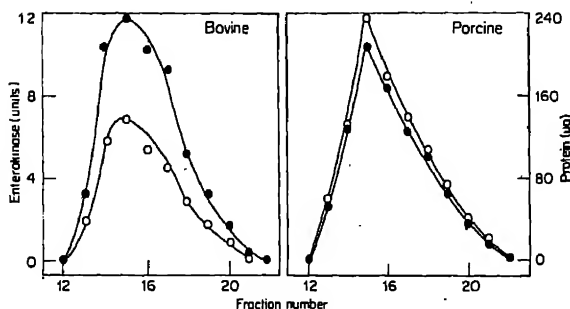
Source of enterokinase	Enzyme units applied on the column	Enzyme units recovered in buffer washings	Enzyme units recovered in acid eluate	Total activity recovered (%)
Bovine	29.6	3.4	22.0	85.8
	59.2	3.4	50.5	91.0
	236.8	24.2	176.5	84.8
Porcine	66.6	9.6	43.5	79.7
	264.4	55.4	161.6	81.1
Human	66.6	31.1	20.9	78.1

enterokinases during affinity chromatography are shown in figure 1. The crude porcine enterokinase was purified 20-fold (specific activity increased from 2.08 to 41.04) and the bovine enterokinase was purified 24-fold (specific activity increased from 3.61 to 85.6) by this single step. The specific activity of the purified human enzyme was 55.5, but the yield was considerably less compared to the bovine and porcine systems.

Typical chromatographic profiles of the fresh, purified bovine and porcine enterokinases on Sephadex G-200 are shown in figure 2. Fairly constant specific activity across the peaks indicated that the preparations are homogenous.



**Figure 1.** Affinity chromatography of crude enterokinase preparations. (o), Protein, (●), enterokinase activity. 59.2 units of bovine enzyme, 66.6 units of porcine enzyme and 66.6 units of human enzyme were subjected to chromatography. Other details are given under materials and methods.



**Figure 2.** Chromatography of purified enterokinases on Sephadex G-200. (o), Protein, (●), activity. Other details are given under materials and methods.

Purified bovine enterokinase and porcine enterokinase on PAGE remained close to the origin as a single band when the run was for 1 h. Increasing the time of electrophoresis upto 4 h resulted in the diffusion and spreading of this band. On SDS-PAGE, both bovine and porcine enterokinases moved as single but diffuse bands (figure 3A and B). The  $M_r$  calculated based on the mobility during SDS-PAGE were 146000 (figure 3A) for the bovine enzyme and 150000 (figure 3B) for the porcine enzyme.

Treatment of the bovine enzyme with 1% of 2-mercapto-ethanol in 10mM

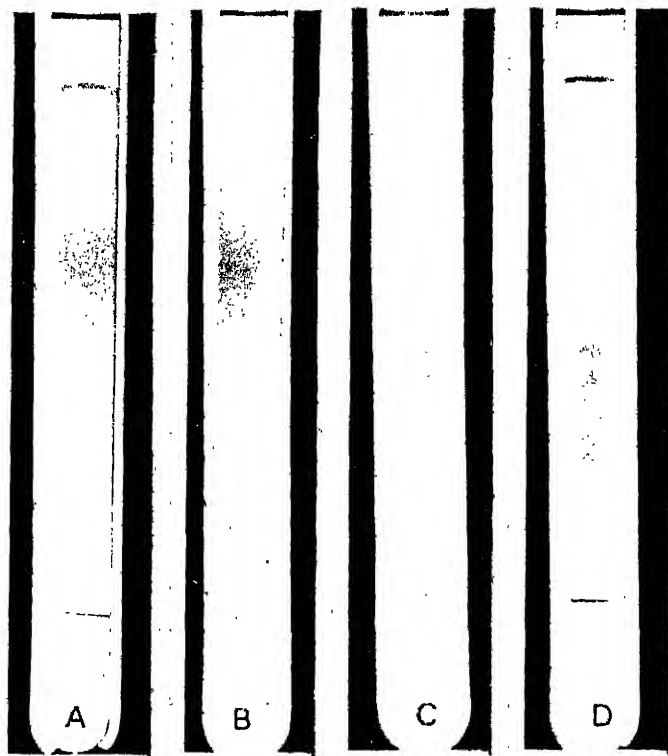


Figure 3. SDS-PAGE of purified enterokinases (A), Bovine enterokinase; (B), porcine enterokinase; (C), bovine enterokinase after treatment with 1% 2-mercaptoethanol for 10 min at 37°C; (D), bovine enterokinase after treatment with mercaptoethanol for 120 min. Other details are given under text.

phosphate pH 7.2 at 37°C for 10 min prior to SDS-PAGE, resulted in two diffuse protein bands with relative  $M_r$  of 34000 and 72000 (figure 3C). Increasing the time of mercaptoethanol treatment to 2 h prior to the application in gel revealed the presence of four major and two minor protein fragments with  $M_r$  of 32 K (32 Kilodaltons), 37 K, 65 K, 78 K, 17 K and 25 K (figure 3D).

### Discussion

Purification of human (Grant *et al.*, 1976), porcine (Grant *et al.*, 1975) and bovine (Anderson *et al.*, 1977) enterokinases by affinity chromatography using immobilized *p*-aminobenzamidine has been reported. On the other hand, failure to achieve adsorption of enterokinase onto insoluble derivatives of *p*-aminobenzamidine has also been reported (Baratti and Maroux, 1976; Barns and Elmslie, 1976). Further *p*-aminobenzamidine being a non-specific inhibitor has also been used for the purification of trypsin (Hixon *et al.*, 1973) and thrombin (Schemer, 1972). Goldberg *et al.*, (1968)

reported that trypsin is adsorbed tightly by intact human epithelium. Pelot and Grossman (1962) have made a similar observation in the rat intestines. In view of this, contamination of enterokinase by trypsin by this purification procedure cannot be ruled out.

Liepnicks and Light (1979) reported the purification of bovine enterokinase using immobilized basic pancreatic trypsin inhibitor. However this inhibitor had no action on porcine enterokinase. In the method reported here both bovine and porcine enterokinases could be successfully purified with good recovery of the enzyme activity. Contamination by trypsin or trypsin-like enzymes cannot arise in this procedure since the kidney bean enterokinase inhibitor had no action on these enzymes (Jacob *et al.*, 1983). However, our procedure could not be used for purifying human enterokinase in sufficient yield since the human enzyme was not retained strongly by the immobilized inhibitor. This is in agreement with the earlier observation that the inhibitor was ten times less active on human enterokinase compared to the bovine counterpart (Jacob *et al.*, 1983). Even with bovine and porcine system, a small proportion of the enzyme was not retained on the column during affinity chromatography. Liepnicks and Light (1979) also observed that 15–40% of bovine enterokinase activity was not retained by immobilized pancreatic trypsin inhibitor.

Our estimated  $M_r$  of bovine enterokinase agrees well with the reported value of 150000 (Liepnicks and Light, 1979). These workers demonstrated that mercaptoethanol treatment cleaved the native enzyme into heavy and light subunits of size 115000 and 35000  $M_r$ , respectively. On the other hand Anderson *et al.* (1977) reported the subunit sizes to be 82000 and 57000. We observed that the treatment with mercaptoethanol for short time resulted in the appearance of two diffused bands during SDS-PAGE with  $M_r$  of 34000 and 72000. An increase in the time of mercaptoethanol treatment to 2 h caused the appearance of four major protein fragments with  $M_r$  of 32000, 37000, 65000 and 78000 and two minor protein fragments with  $M_r$  of 17000 and 25000. These discrepancies could be due to the fact that the native enzyme is made of two subunits of different sizes linked by S-S bridges. At least one of the subunits could in turn be made up of smaller peptides linked by disulphide linkages. Differences in the conditions of reduction may be responsible for the selective cleavage of S-S bonds resulting in the formation of a number of peptide fragments.

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## Cytophotometric manifestation of the biochemical changes in various skin constituents induced by a single subcutaneous administration of 7,12-dimethylbenz(a)anthracene in rabbit

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**Abstract.** The effect of a single subcutaneous administration of 7,12-dimethylbenz(a)anthracene, the powerful complete carcinogen, under the skin was studied in the rabbit. The study reports an ordered sequential biochemical and cytophotometric changes induced by the carcinogen. While the biochemical studies comprised of sequential quantitative estimations of DNA, RNA and protein per mg of skin, the cytophotometric studies consisted of the estimation of the level of macromolecules in a cell/nucleus, in different skin constituents viz. epidermis, hair follicle shaft region and hair follicle bulb region. Biochemical results indicate an initial rise in the level of DNA and RNA and reduction in protein upto 20 days. From 40 to 60 days treatment duration there was a 'steady-state' showing a constant level of all the parameters while the highest peak was observed on the 80th day. The site of these biochemical changes among different skin constituents was determined with the help of cytophotometer which indicates the highest level of nucleic acids in epidermis region right from the initial stage (i.e. 10th day) to the 90th day of treatment in comparison to two other regions hair follicle shaft and hair follicle bulb regions. Histological studies, on the other hand, reveal a greatly, though gradual, increased nuclear area and the highest rate of proliferation only in hair follicle bulb region, thus suggesting a definite role of this region of the skin in the carcinogenesis. All these results suggest that the important event in the initiation phase of 7,12-dimethylbenz(a)anthracene mediated skin carcinogenesis in rabbit might be associated with epidermal region but the role of hair follicle bulb region should also be considered as of an equal significance during the process.

A conspicuous difference in the behaviour of rabbit skin constituents has been noted when the results of the study are compared with the earlier reports on mice.

**Keywords.** Carcinogenesis; cytophotometry; skin; 7,12-dimethylbenz(a)anthracene; hair follicle.

### Introduction

The skin of the mouse has been the tissue of choice for a long time for the quantitative estimations of DNA, RNA and protein during the promotion phase of tumour (Baird *et al.*, 1971; Raick *et al.*, 1972; Balmain *et al.*, 1977). With the help of cytophotometer one can estimate total and per unit area contents of the macromolecule in a single cell if the precise area of the cell is known (Shah *et al.*, 1975). However, histochemically prepared slides are required in order to obtain contents of the metabolite in the cell. Since any comparative study on the role of different skin constituents in induced skin

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Abbreviations used: DMBA, 7,12-Dimethylbenz(a)anthracene; s.c., subcutaneous; Ep, epidermis; HS, hair follicle shaft; HB, hair follicle bulb.

tumour in rabbit has not yet been made, we designed this study using biochemical and cytophotometric techniques to compare the results obtained by these two procedures and to study the sequential changes in different skin constituents after a single subcutaneous injection of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) in the rabbit.

The aim of the present study was to investigate a possible basis for the species difference in skin carcinogenesis in rabbit and mouse and to establish a correlation between biochemical and cytophotometric alterations in order to evaluate the most active site among all the skin constituents during the initiation and promotion phases when DMBA was administered subcutaneously.

### Materials and methods

Healthy male albino rabbits were divided into two groups—control and treated. While the animals of control group received only 0.1 ml of olive oil subcutaneously, animals of treated group received a single subcutaneous (s.c.) injection of 1.5 mg DMBA in 0.1 ml olive oil without subsequent use of any promotor. This dose level was found to be capable of inducing all preneoplastic and neoplastic changes in the skin of the rabbit and tumours appeared at the site of treatment after 100 days of treatment. One separate group of control animals was run with each treated group. The day of carcinogen exposure was considered as zero day and after an interval of every 10 days one group of treated and corresponding control animals was sacrificed and the skin from the treated site was excised for the study. Thus, the first group of treated and control animals was sacrificed on the 10th day and the last on the 90th day. One part of the excised skin was fixed separately in Bouin's and Carnoy's fixatives for histological and histochemical preparations while another part of the tissue frozen immediately in ice for biochemical estimations of protein (Lowry *et al.*, 1951), DNA and RNA (Ceriotti, 1952, 1955).

Slides prepared by various standard histochemical techniques for the localization of DNA (Feulgen and Rossenback, 1924), RNA (Tepper and Gifford, 1962) and protein by mercuric chloride-bromophenol blue method as suggested by Pears (1968) were used for the cytophotometric studies of preneoplastic lesion. Control slides were prepared by treating the slides with the enzymes DNase and RNase. The extinction value, total and per unit area quantity of the macro-molecules were estimated in the cells in epidermis (Ep), hair follicle shaft (HS) and hair follicle bulb (HB) regions of skin by the method of Shah *et al.* (1975) in all treated and control animals. For histological observations 6  $\mu$  thick paraffin sections were stained with haematoxylin and eosin. Nuclear and cellular areas were estimated in all the three regions of the skin by putting an ocular meter in the eye piece of the microscope.

### Results

#### *Microscopic observations*

Untreated rabbit skin has a 1–2 nucleated cell thick Ep. Hair follicles were either resting or growing actively. The dermis, consisting of strands of collagen, was strongly

eosinophilic. A sequence of changes in the epidermis and the dermis was observed after s.c. administration of DMBA. Focal cellular hyperplasia of epidermis was seen after 20 days and in these areas the epidermis was piled up in 4-5 cells in thickness. Keratinization and epidermal thickness was found to be increasing corresponding to increased duration of carcinogen exposure and after 60 days the Ep was quite thickened and highly cellular. Hair follicles also exhibited moderate increase both in number and cellularity after 20 days. Epithelial sheath of the hair follicles displayed cellular hyperplasia in which crowding of the cells could be seen in or around the appendages. Grouping of the hair follicles was also noticed after 20 days and each group as seen on the 80th day was consisted of nearly 17 hair follicles. The number of hair follicles per unit area of the skin was increased by 6 times where as only 3 fold increase was observed in the number of epidermal nucleated cells after 90 days of treatment.

Sequential changes in the area of cells and nuclei in various skin components are shown in figures 1 and 2. Initially, there was a slight increase (app. 20%) in the cellular

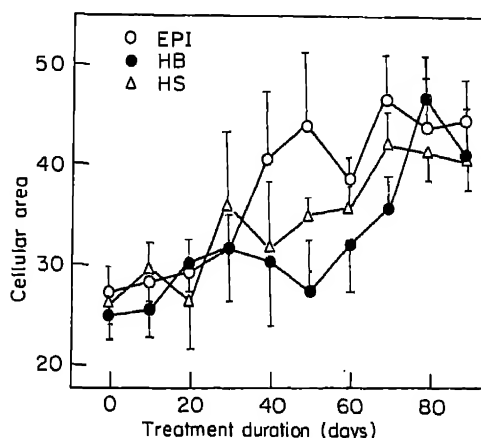


Figure 1. Changes in cellular area in various regions of skin.

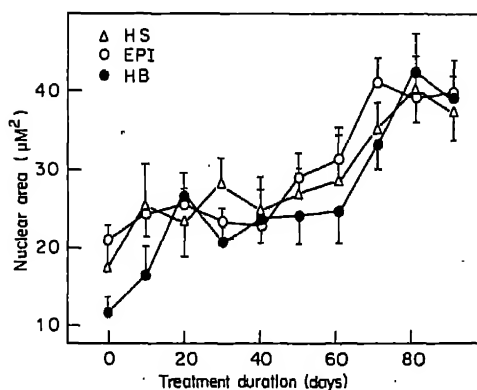


Figure 2. Changes in nuclear area in different regions of skin.

area of the HB region cells on the 20th day after the treatment, while the Ep and HS did not show any change in the cellular area. The maximum cellular area in Ep and HS regions was detected on the 70th day and on the 80th day in the case of HB region which was evident by a 0%, 60% and 85% increase respectively in Ep, HS and HB regions after the treatment. The area onwards was reduced on the 90th day (figure 1).

Increase in nuclear area, on the other hand, was maximum in HB region after 20 days of treatment. In HB region there was about 145% increase in nuclear area while about 25% and 28% increase was found in Ep and HS regions respectively. The highest peak of the nuclear area, observed on the 70th day in Ep region and on the 80th day in HS and HB regions was evident by a 90%, 123% and 264% increase respectively in the three regions. On the 90th day the nuclear area was reduced in all the constituents (figure 2).

### Biochemical changes

Quantitative biochemical changes in DNA, RNA and protein level are shown in figure 3. A continuous increase in the level of RNA from 0 to 30 days and a reduction thereafter on the 40th day was found after the treatment. From 40 to 60 days treatment duration there was a steady state showing a constant level of RNA, followed by a spurt of increase upto the 80th day. On the 80th day RNA per mg tissue was increased by 500% compared to the control level.

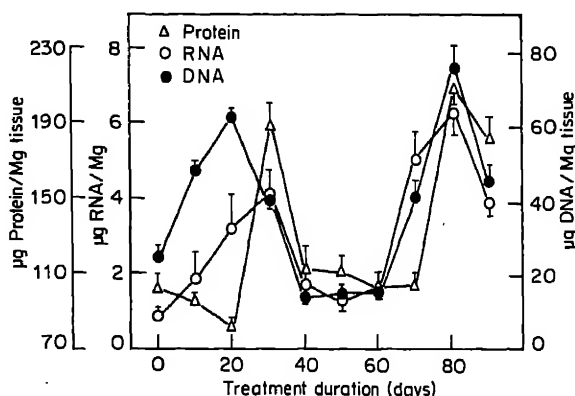


Figure 3. Quantitative changes in nucleic acids and protein after DMBA treatment.

In the case of DNA an initial enhancement in the level was observed upto 20 days of treatment followed by a rapid fall upto 40th day. From 40 to 60 days the DNA level per mg of tissue remained unchanged and increased again from 60th to 80th day with about 160% increase in the level in comparison to control level. The DNA level reduced again on the 90th day.

Observations on protein indicate that there was a slight depletion (about 20%) in the level from zero to 20th day after the treatment. A sharp enhancement, about 85% of the control level, however, was found on the 30th day after the treatment. A steady state, similar to DNA and RNA, was also reported in the case of protein during 40 to 70 days

treatment duration. The maximum level of protein with a 100% increase was reported on the 80th day.

### Cytophotometric observations

Changes in total contents and concentration per unit area of DNA, RNA and protein in the cells/nuclei in Ep, HS and HB regions as studied with the cytophotometer are described below.

**Ep:** A sharp increase i.e. about 7 and 3 times in the level of both total contents and per unit area concentration of DNA in Ep region was observed during the first 20 days treatment duration which remained almost unchanged till the 50th day of treatment. From 50 to 80 days treatment duration the level of the total contents of DNA increased significantly in the nuclei. Hence, the total contents and concentration per unit area of DNA observed after 80 days of treatment period was about 20 and 4 times respectively of the control level (figures 4 and 5).

A continuous increase upto 80 days of treatment duration was observed in total RNA contents in the cells of Ep region. The concentration of RNA per unit area of the cell, on the other hand, increased initially upto 30 days and then from 40 to 80 days after the treatment. On the 90th day a rapid fall was observed both in the total RNA contents and concentration per unit area of the cell.

A remarkably decreased level of total contents and concentration per unit area of protein was observed on the 10th and 20th day respectively in the initial stages of carcinogenesis after the treatment. From the 10th day onwards the total contents of protein increased gradually showing about a 75% increase in the level on the 80th day in comparison to control level. Similarly, though the level of per unit area concentration of protein in the cell was also observed to be increasing gradually from 40th to 80th day, it remained quite low in comparison to control level. On the 90th day

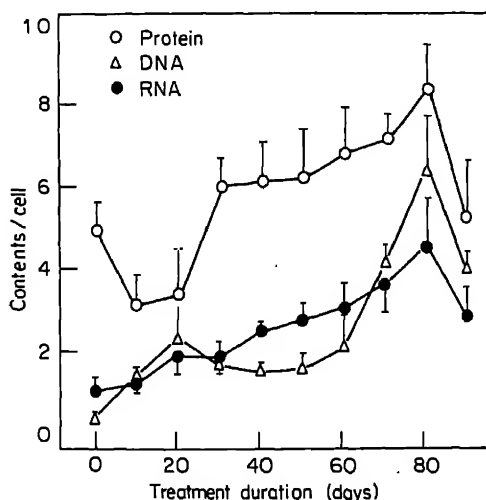


Figure 4. Changes in total contents of metabolites in epidermal region.

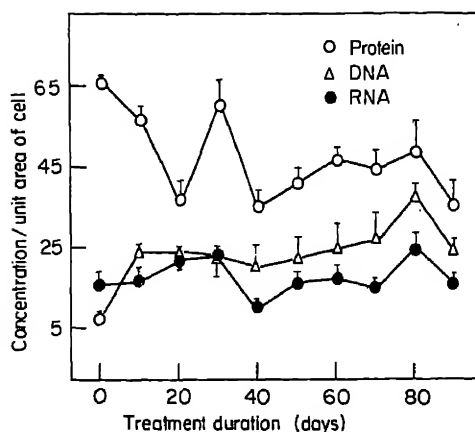


Figure 5. Changes in concentration per unit area of metabolites in epidermal region.

there was again a sharp reduction in the level of both total contents and concentration per unit area of protein in the cell.

**HS region:** The level of total contents of DNA in the nuclei of HS region cells increased by more than 10 times within 20 days after the treatment; while the changes in the total contents of DNA were marginal during 20 to 60 days treatment duration, a spurt in the level was found during 60 to 80 days treatment period. DNA level as observed on the 80th day was about 40 times of the control level which again decreased on the 90th day (figures 6 and 7).

Any notable change in the level of total RNA contents of the cell could not be observed till the 40th day after the treatment but during the period of 40 to 80 days of

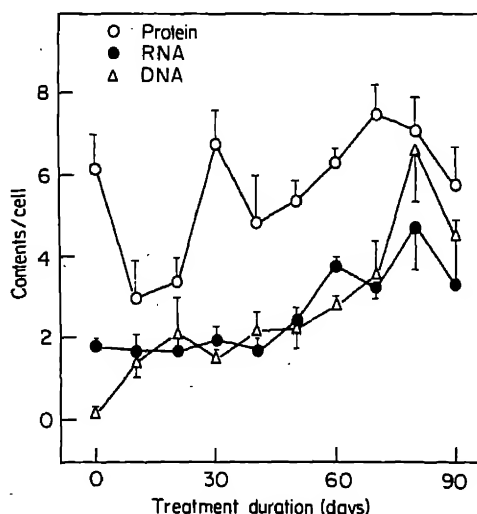


Figure 6. Changes in total contents of metabolites in shaft region of hair follicle.

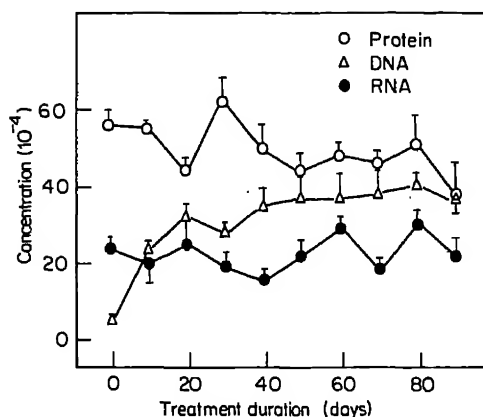


Figure 7. Changes in concentration per unit area in HS cells.

treatment the level of RNA contents was increased by 150 % of the control level. Similar to total contents, the concentration per unit area also remained unchanged till the 40th day after the treatment and increased from 40 to 60 days duration. About 75 % increase in the level was reported on the 60th day. Concentration per unit area then decreased on the 70th and 90th days.

The total contents of protein in the cell got reduced significantly on the 10th day and subsequently increased till the 30th day after the treatment. A gradual elevation in protein contents in the cell was again found during the period of 40 to 70th day, reaching its highest peak, though the level was only slightly higher (20 %) than the control level. On the other hand, concentration per unit area was slightly lesser (app. 10 %) on the 80th day, it reduced by 35 % on the 90th day in comparison to the control level after the treatment.

**HB region:** The total contents of DNA per cell in the HB region increased by 4 times of the control level after the treatment for 20 days. From 20 to 40 days of treatment duration the level remained unchanged but a ten fold increase in total contents of DNA per cell was found on the 80th day. DNA concentration per unit area initially increased upto 30th day and reduced subsequently till the 80th day reaching again the control level (figures 8 and 9).

About four fold elevation in the level of total contents of RNA in the cells of HB region was found on the 80th day followed by a slight reduction on the 90th day after the treatment. Concentration of RNA per unit area in the cell did not exhibit any alteration in the level till the 40th day but increased from 40 to 70 days and reduced subsequently on the 90th day.

Following the DMBA treatment total protein contents and concentration of protein per unit area reduced in the beginning till the 20th day and increased on the 30th day. The highest peak with 43 % elevation on the 80th day was found in the level of total protein contents while a 67 % reduction was reported in the level of protein concentration per unit area in the cell on the 90th day after the treatment.



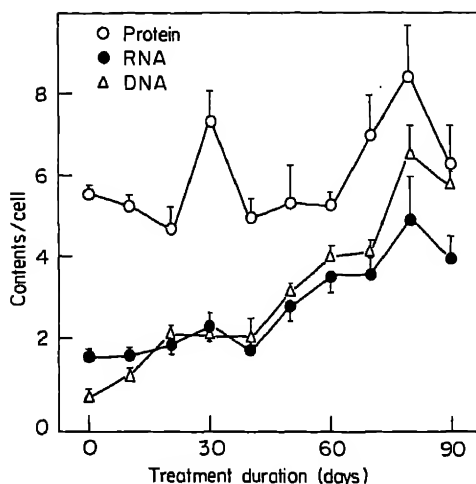


Figure 8. Changes in total contents of metabolites in bulb region of hair follicle.

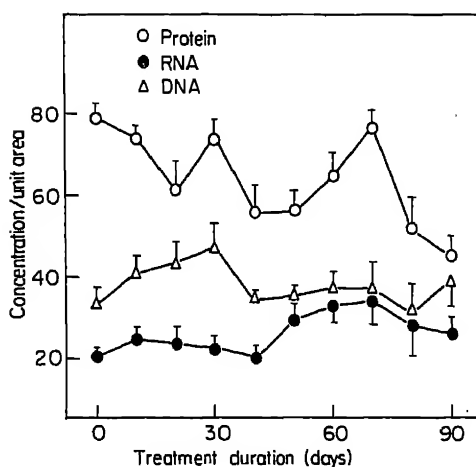


Figure 9. Changes in concentration per unit area of metabolites in bulb region of hair follicle.

## Discussion

Though many workers have discussed in detail the role of the epidermis in tumour induction (Slaga *et al.*, 1974; Wiebel *et al.*, 1975; Boutwell, 1976) reports are not available examining the significance of bulb region during the carcinogenesis. Thus, specific attention has been paid in this study towards the HB region in order to investigate its role in carcinogenesis in the rabbit with the help of biochemical as well as cytophotometric techniques. Hence, this study is an attempt to establish a correlation between the biochemical and cytophotometric quantitative alterations and to in-

investigate for the first time the role of all the skin constituents separately during the skin carcinogenesis in the rabbit when the carcinogen is injected subcutaneously.

It was found that any change in the level of macromolecule was in fact a net change in various skin constituents determined by a relative study of the changes in total and per unit area contents of the macromolecules and the nuclear/cellular area. Since the binding between carcinogen and nucleic acids, occurring in the initiation phase of carcinogenesis in cell (Miller, 1951; Miller and Miller, 1966, 1974; Boyland and Green, 1962; Heidelberger, 1970) is known to stimulate the synthesis of nucleic acids (Tominga *et al.*, 1970; Henning and Boutwell, 1970; Paul, 1969; Balmain *et al.*, 1977), it can be suggested that the quantitative increase in the level of DNA and RNA on 20th day after DMBA treatment represents a stimulatory action of the carcinogen on the nucleic acids. During the same period reduction in the quantitative level of protein is a consequence of a net reduction in protein synthesis which is evident by the reduced level of total contents and concentration per unit area of protein in all the regions of the skin. Similarly during 30 to 60 days of treatment duration the level of DNA and RNA per mg of tissue was found almost unchanged. During this period it was found cytophotometrically that there was only a marginal increase in the level of concentration per unit area and total contents of the metabolites (*i.e.* DNA and RNA) in all skin constituents along with an insignificant increase in the nuclear area. Hence, any significant change in biosynthesis and the level of the nucleic acids could not be observed during this period. The steady state of protein observed during 40 to 70 days of treatment duration can also be explained on a similar ground with the help of cytophotometer. The highest peak of the levels of all the macromolecules as estimated per mg of tissue, was found on the 80th day after the treatment. At the same time the level of total contents and concentration per unit area of macromolecules in a cell were also found to be highest. All these observations thus, suggest the significantly increased synthesis of nucleic acids and protein in all the skin constituents at this stage.

In addition, to explain the site of biochemical changes in skin constituents, cytophotometer was used to compare the rate of metabolism among all the skin compartments. Since the rate of metabolism is greatly associated with the size of the nucleus, nuclear volume has been found to be changing in correspondence with the rate of metabolism in the cell (Jacobj, 1925). Hence, the cells with large nuclei are observed in liver lobes particularly in the intermediate part of the lobule (Jacobj, 1925), where the circulation takes place under the optimal conditions (Clara, 1930) and in the area of growth in the culture of fowl fibroblast (Bucher and Gattikar, 1950; Bucher, 1951). Though the highest DNA level, an index of highest rate of DNA synthesis (Tominga *et al.*, 1970; Balmain *et al.*, 1977), was observed in Ep region among all the skin constituents, the maximum increase in the nuclear area was observed only in HB region. A 145% increase in nuclear area in HB region on the 20th day in comparison to 25% and 28% increase in HS and Ep regions and a 234% increase in HB region in comparison to 117% increase both in HS and Ep regions after 90 days is reported in this study. Further, the fastest rate of cell proliferation was observed in HB region which is evident by the growth of HB cells in the dermal region only after 20 days and a significant increase (app. 6 times) in the number of hair follicles per unit area of skin after 90 days. All these results indicate an equally significant role of HB region in inducing the skin tumour in the rabbit though the Ep region might be the site of

interaction of carcinogen with DNA and increased synthesis of nucleic acids.

In a similar study in mice, Bhisey *et al.* (1979) have reported an increased level of RNA and protein and reduction in DNA in all the skin constituents. Contrarily, a significant increase in DNA and RNA and reduction in protein in the initial stages was observed in this study, thus indicating a different kind of behaviour of the skin of the rabbit than in that of the mice which is probably due to a difference in skin thickness (Garg *et al.*, 1982).

To conclude, a single s.c. injection of DMBA in the rabbit can cause the preneoplastic changes in the Ep as well as in the hair follicles which are reflected biochemically by increased level of DNA, RNA and protein in all skin constituents after 90 days. All these alterations can be correlated well with the cytophotometric studies. While the biochemical and cytophotometric studies indicate an important role of the Ep in initiation of tumour, histological studies reveal that the site of active metabolic alterations must be the HB region. It is, therefore, suggested that both the skin regions *i.e.* Ep and HB play an equally significant part in inducing the tumour in the rabbit skin. However, a conspicuous species difference in the behaviour of the skin in rabbit and mice has been observed in this study.

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## Characteristics of fluorescamine modified bacteriorhodopsin

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**Abstract.** The light activated proton pump, bacteriorhodopsin was modified with varying amounts of fluorescamine, the fluorescamine to protein ratio ranging from 1 to 100. The modified protein was washed free of excess of fluorescamine and reconstituted into phospholipid vesicles to check the proton pumping activity. Although the spectral investigations indicated chemical modification, the circular dichroism measurements pointed to an overall loss of the trimeric structure of the protein. The implications of the present study are that the modifying agent can interact non-specifically with the protein, altering its structural parameters, which in turn affects the function of the protein.

**Keywords.** Bacteriorhodopsin; fluorescamine; reconstitution; vesicles; proton pump.

### Introduction

Bacteriorhodopsin (bR), the light activated proton pump of *Halobacterium halobium*, is a deceptively simple protein (Stoeckenius *et al.*, 1980). According to different models, various aminoacid residues have been implicated in the proton pumping activity (Eisenbach *et al.*, 1979). However, chemical modification of these residues at the site of the labile proton is an elegant method to exclude or include any particular set of aminoacid residues in the mechanism.

Fluorescamine (FL), is an amine specific reagent (Udenfriend *et al.*, 1972) which could selectively modify surface amino groups such as those of lysine (Tu, 1979). Our earlier paper (Tu *et al.*, 1981) deals with effect of FL on reconstituted bR. However, controversies exist regarding the role of lysines in the proton pumping activity. Lemke *et al.* (1982) concluded that the acetylation of lysines did not inactivate the protein, contrary to the observation made by Maeda *et al.* (1982) and Takeuchi *et al.* (1981). The present study was undertaken to resolve this controversy.

### Materials and methods

FL was purchased from Sigma Chemicals Co., St. Louis, Missouri, USA. bR was isolated and assayed according to the procedure published earlier (Tu *et al.*, 1981; Ramirez *et al.*, 1982). All other chemicals used were of the best quality available.

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Abbreviations used: bR, Bacteriorhodopsin; FL, Fluorescamine; CD, circular dichroism; UV, ultra-violet.

### Modification of the protein by FL

The purple membrane sheets were suspended in borate buffer pH 8.8, to a concentration of 500  $\mu\text{g}/\text{ml}$ . Required amount of the FL solution in acetone was added; the amount of acetone was equalized in all tubes. The tubes were incubated at room temperature for 15 min. The protein was sedimented by spinning at 100,000  $g$ . The pellet was washed three times with deionized water and finally suspended in 75 mM KCl, 1 mM Hepes, pH 8.0 to a concentration of 500  $\mu\text{g}/\text{ml}$ . A part of this final suspension was used for reconstitution into sonicated lipid vesicles (Ramirez *et al.*, 1981; Tu *et al.*, 1981) and the rest reserved for the spectral investigation and amino acid analysis.

The ultra-violet (UV) and visible spectra were scanned in a Cary 118 double beam spectrophotometer. The circular dichroism (CD) spectra was monitored by a JASCO J20 spectropolarimeter and the fluorescence spectral measurements were carried out in a Perkin Elmer model MPF 44 instrument.

### Reconstitution and assay of the protein

The reconstitution of the purified protein into sonicated phosphatidyl choline vesicles was achieved by incubating the mixture overnight at 0°C in presence of excess of octyl glucoside. The protein incorporated vesicles were spun down at 100,000  $g$  and resuspended in 150 mM KCl to yield a final concentration of 500  $\mu\text{g}/\text{ml}$ . 2 ml of this suspension was introduced into a Gilson Medical oxygen chamber equipped with a pH electrode. Conditions of zero membrane potential were established by adding excess valinomycin (500 ng). A 750 W slide projector served as the light source. The reaction chamber was thermostatted at 25°C by circulating water through the outer jacket.

### Aminoacid analysis

The modified and unmodified samples were hydrolysed for 24 h at 110°C with 5.7 N HCl containing phenol (0.05 %) in sealed evacuated tubes. The analyses were carried out on a Beckmann 119CL aminoacid analyzer using the standard protein hydrolyzate program (ca. 90 mn/run). The samples and standards, over 100 runs were performed. The standard analysis was 10 nano mol of each aminoacid.

### Results

FL is known to react exclusively with free primary amino groups (Udenfriend *et al.*, 1972). Kushwaha *et al.* (1975) have reported that the lipids of purple membrane do not contain primary amino groups. Thus when purple membrane sheets are treated with fluorescamine the interaction is possible only on the  $\alpha$ -amino group of the lysine side chain (Tu *et al.*, 1981). According to the recent model by Khorana's group (Huang *et al.*, 1982), of the seven lysines in bR, six (30, 40, 41, 129, 159 and 172) are peripheral with their amino groups free. The seventh, lys 216 carries the chromophore retinal on the

amino group as a Schiff base and is buried inside the helical cavity. Thus all the other six lysines are modifiable by FL, especially since the modification is being carried out at the purple membrane level, before reconstituting into lipid vesicles. The purple membrane contains 80% bR and 20% lipids. As the lipids of purple membrane do not contain amino groups, one would expect the modification to be virtually complete with a FL to bR mol ratio of 10:1. Under the conditions of the present study, amino acid analysis showed only a maximum of 35–40% modification. Earlier Chen *et al.* (1978) have observed that about 100 fold excess of FL is required for 80–90% completion of reaction with primary amines.

The proton pumping activity of the modified protein reconstituted into sonicated phosphatidyl choline vesicles is shown in figure 1. The profile suggests a two stage decay. The initial pumping rate  $R_0$  decreases to approximately 60% with the first ten mol of FL. The decrease is much slower with further increments of FL. It should be noticed here that at an FL/bR value of 50, the activity falls to about 20% of the original value. Above this ratio the activities were too low to be assayed and reproduced accurately. However, the pumping activity was nil above a ratio of 100.

The modified protein has an intense absorption peak at 560 nm (Stoeckenius, 1980). Figure 2 presents the spectra of the modified samples along with that of the control. The perturbations on the 570 nm peak are not serious; only a slight broadening and a small decrease in intensity results. Hence this peak of the protein at intermediate concentrations of FL are not shown due to excessive overlap. The emergence of a new peak at 390 nm which increases in intensity as the FL/bR ratio is the interesting feature of these spectra.

FL is known to form intensely fluorescent covalent derivatives with primary amines. It is by virtue of this characteristic that this reagent has been recommended for the fluorometric assay of proteins in the nanogram range (Udenfriend *et al.*, 1972; Bohler *et al.*, 1973). These derivatives of FL have an absorption in the 370–390 nm region and an emission peak in the 450–480 range (Chen *et al.*, 1978). Earlier Lam *et al.* (1980) have observed the 390 nm peak in FL-bR systems and they have attributed this to be the amino FL covalent linkage. The fact that this peak continues to increase in intensity even after a FL/bR mol ratio of 100 substantiates the incompleteness of modification. In an attempt to completely characterise the 390 nm peak we scanned the fluorescence

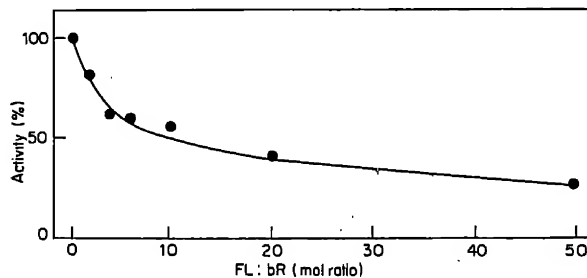
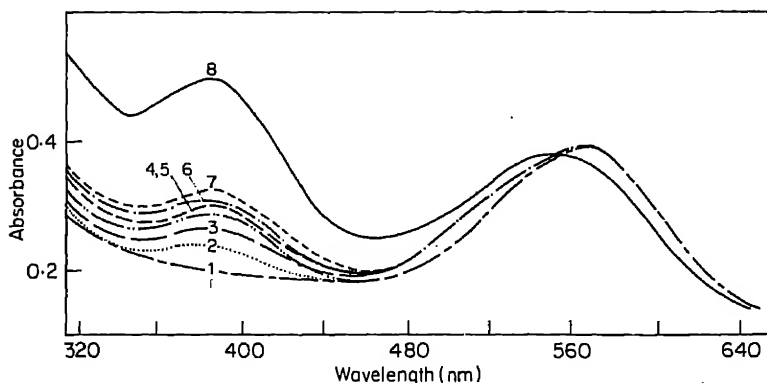


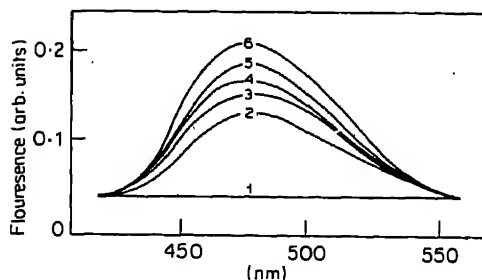
Figure 1. Effect of FL modification on the proton pumping activity of bR. The rate in unmodified bR taken as 100 = 948 nmol  $H^+$ /mn mg protein. The assay procedure is given in materials and methods.





**Figure 2.** Absorption spectra of FL modified bR. Samples 1 to 7 have the following bR to FL ratio.

1. Unmodified sample; 2. 1:2; 3. 1:4; 4. 1:6; 5. 1:10; 6. 1:20; 7. 1:50; 8. 1:100.  
The assay procedure is explained in the text.

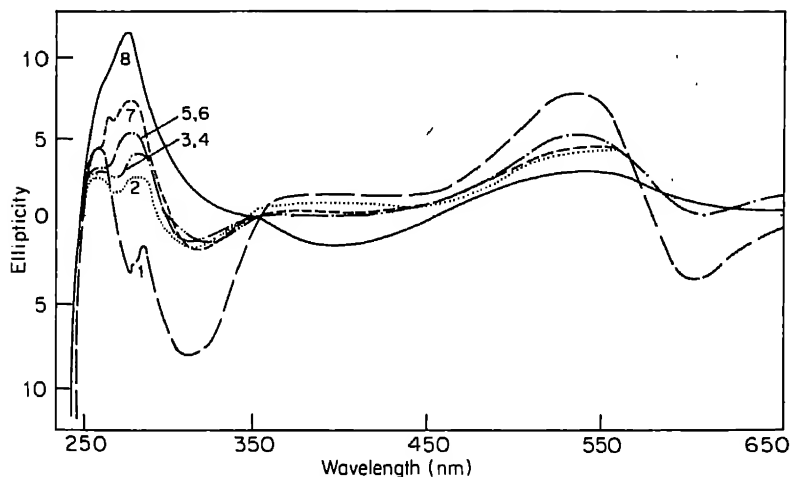


**Figure 3.** Fluorescence spectra of modified bR. The protein was modified with FL in borate buffer as described in the text. The samples have the following bR to FL mol ratios.

1. Unmodified; 2. 1:10; 3. 1:20; 4. 1:50; 5. 1:100; 6. 1:200.

spectra by exciting at 390 nm. Figure 3 presents the related data for FL-bR system. A sharp emission band appears at 470 nm and increases in intensity with increase in the FL concentration. These data show covalent modification of the protein by FL, at the same time indicating the incompleteness of the reaction.

Figure 4 summarises the CD spectra, which throws light on the structural features of bR under the conditions of modification. The visible CD spectrum of unmodified bR consists of intense positive and negative lobes with a cross over point at 570 nm (Becher *et al.*, 1976; Heyn *et al.*, 1975). Based on experimental evidence, they have attributed this feature of CD in the visible region to an exciton coupling between the retinal chromophores and adjacent protein molecules held in a rigid hexagonal lattice. The bands in the near UV region are the aromatic CD bands. bR has tyrosines, and tryptophan but no cystines. It is established that the near UV CD bands reflect the tertiary structure, whereas the far UV CD bands usually provide information about the secondary structure (Strickland 1974). In the present system of FL-bR, the negative peak around 600 nm disappears and the positive peak around 520 nm broadens



**Figure 4.** CD spectra of FL modified bR. The protein was modified with FL in borate buffer as described in the text. The samples have the following bR to FL ratio.

1. Unmodified; 2. 1:2; 3. 1:4; 4. 1:6; 5. 1:10; 6. 1:20; 7. 1:50; 8. 1:100.

considerably. Also the doublet peak (268 and 286 nm) of the unmodified protein merges into a single 280 nm peak in the modified sample. The loss of the characteristic features of the CD spectra could be due to a loosening of the rigid lattice of the bR cluster.

## Discussion

At this point three pieces of information arising out of the present study have to be correlated; (i) the initial rate of photo-activated proton pumping decreases with increasing mol ratios of FL/bR, (ii) the visible and fluorescence spectra record increasing modification, and (iii) the CD measurements denote a loss in the structural integrity of the protein. Campos-caviers *et al.* (1979) studying the modification of the tyrosine residues of bR with nitromethane made essentially the same observations. They have suggested denaturation of protein at higher extends of modification. The significance of the rigid arrangement of bR clusters for its functionality has been pointed out by Becher *et al.* (1976) and Heyn *et al.* (1975). They observed that any perturbation, physical or chemical which induces a rotational mobility or a disorder made the CD spectrum vanish. Strickland (1974) also points out the three factors that influence the intensities of aromatic CD bands, (i) the rigidity of the structure, (ii) interaction of aromatic ring with its neighbours, and (iii) no. of aromatic residues.

More significantly, Lam *et al.* (1980) studying the effect of FL-modification on the structural parameters observed that the modified samples acquire an "enhanced mobility of the chromophore". They also reported the CD experiments which suggested a loss of exciton coupling between the molecules in the hexagonal arrays of bR trimers. They however, did not measure the light induced proton pumping. It is entirely possible that the deterioration of the pumping activity of bR is due to two isolated events brought about by a large excess of fluorescamine in the medium: (i) the

actual modification, and (ii) a slow deterioration of the tertiary structure of the protein by nonspecific interaction with excess FL present in the system.

To interpret the results of the present work, we assume that FL can interact with the protein at two different levels. In the initial stage within a FL to bR ratio of 10, at least one lysine is modified bringing about a sharp drop in  $R_0$ . Further increments of FL fail to cause uniform increase in covalent modification as suggested by amino acid analysis. The spectral data are also in corroboration with this possibility. As the concentration of FL rises in the medium, a second possible consequence, which is the implantation of FL between the helical cavities, become apparent. This possibility of FL has been documented by an earlier work on mitochondrial protein (Lam *et al.*, 1980). This implantation directly or indirectly alters the structural parameters of the protein, as suggested by the CD data and this alteration impairs the ability of the protein to respond to the light signal in full capacity.

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## Inhibition of methanogenesis and its reversal during biogas formation from cattle manure

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**Abstract.** The composition of volatile fatty acids in the biogas digester based on cattle manure as substrate and stabilised at 25°C showed that it contained 87-88% branched chain fatty acids, comprising of isobutyric and isovaleric acids, in comparison to 38% observed in the digester operating at 35°C.

Mixed cellulolytic cultures equilibrated at 25°C (C-25) and 35°C (C-35) showed similar properties, but rates of hydrolysis were three times higher than that observed in a standard biogas digester. The proportion of isobutyric and isovaleric were drastically reduced when C-25 was grown with glucose or filter paper as substrates. The volatile fatty acids recovered from C-25 (at 25°C) inhibited growth of methanogens on acetate, whereas that from C-35 was not inhibitory. The inhibitory effects were due to the branched chain fatty acids and were observed with isobutyric acid at concentrations as low as 50 ppm.

Addition of another micro-organism *Rhodotorula* selected for growth on isobutyric completely reversed this inhibition. Results indicate that the aceticlastic methanogens are very sensitive to inhibition by branched chain fatty acids and reduction in methane formation in biogas digester at lower temperature may be due to this effect.

**Key words.** Methanogenesis; methane inhibition; isobutyric; isovaleric; biogas; volatile fatty acids; *Rhodotorula*.

### Introduction

Conversion of cellulosic manure to methane is a complex multistep process which operates under highly reducing conditions involving a large number of microorganisms. Basically it involves converting cellulose and other polymers in the biomass to fatty acids and then to methane (Chen *et al.*, 1980; Zeikus, 1980; Mackie and Bryant, 1981). Under any given set of conditions, interaction between these microbial groups determine overall rate of methane formation and stability of the process. In rumen as well as in biogas digester, most of the methane is formed from acetate by aceticlastic methanogens whose metabolic activity is quite responsive to concentration of fatty acids. It is known, for instance, that concentration of volatile fatty acids (VFA) in excess of 2000 ppm inhibits methane formation in anaerobic digester (Chen *et al.*, 1980; McCarty and McKinney, 1961; Kroeker *et al.*, 1979). In addition, the entire process of biogas formation is sensitive to changes in temperatures, and it is a common experience

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Abbreviations used: VFA, volatile fatty acids; IBA, isobutyric acid; IVA, isovaleric acid; TVM, total volatile matter, TS, total solids.

that the methane production is greatly reduced when the temperature falls below the ambient (25°C).

In this communication, we have presented evidence to show that, in addition to the concentration of VFA in the digester, its composition is equally important in determining inhibitory effects on methane formation. At lower temperatures (25°C), there is a significant accumulation in the digester of branched chain fatty acids such as isobutyric (IBA) and isovaleric (IVA) which inhibit methane formation. Experiments indicate that this inhibition is at the level of aceticlastic methanogens growing on acetate and can be reversed by incorporating another micro-organism which uses branched chain fatty acids for growth, suggesting an approach to improving the methane yields and making the system more tolerant to temperature changes.

## Materials and methods

### *Enrichment of cellulolytic cultures*

Mixed cellulolytic cultures were established in a 5 L capacity anaerobic fed-batch digester (working vol 4 L) fitted with an inverted tube for collecting gas over 0.1 N HCl from which it was continuously tapped for GLC analysis of methane and carbon dioxide. Sources of organisms were buffalo dung (15–19% solids) and rumen fluid. This was diluted twofold wt/wt and fed to the two digesters, one operated at 25°C and the other at 35°C for 10 weeks with an average retention time of 40 days. During this period, the culture was stabilised as determined by constant rate of methane and VFA formation. Mixed stabilised cultures were then transferred to the medium described by Hobson (1957) for cellulolytic anaerobes with only one variation in that filter paper (0.6 g) was used in place of cellulose powder. The cultures were then transferred once every fourth day in a batch digester (working vol 4 L) at 25°C and 35°C. They were labelled as C-25 and C-35 respectively and used as an inoculum for other digesters used in the study.

### *Enrichment of methanogens*

Biogas digesters were set up as above at 25°C and 35°C for 10 weeks using cattle manure as substrate. At the end of 10 weeks, 1% sodium acetate and 0.5% methanol were added once every third day along with fresh cattle manure (2.5 g solids per litre). Digesters were operated for 4 more weeks and the mixed cultures stabilised at temperatures (25°C or 35°C) and pH 6.8 were then transferred to the medium for methanogens. The mixed culture, so stabilised, converted about 80–85% of acetate to methane.

### *Anaerobic culture techniques*

The anaerobic culture techniques described by Hungate (1966) and modified by Miller and Wolin (1974) were strictly followed for both cellulolytic and methanogenic bacteria.

For studies with cellulolytic cultures, fed-batch laboratory digesters were set up at 25°C and 35°C with a capacity to handle 1.6 kg of 50% slurry of the cattle manure (8% total solids) and was replenished with 20 g of fresh manure (1.6 g total solids) every third

day. The cattle manure thus to be used as substrate and for subsequent additions was sterilised by autoclaving at 15 psi for 15 min. Inoculum C-25 and C-35 prepared as described above were added at 10% level. Quantity of VFA and total reducing sugars were monitored in effluent every day. Effluents from digesters operating at 25°C and 35°C were collected, centrifuged at 7800 *g* and sterilised by passing through Millipore membrane filters. Filtrates labelled VFA-25 and VFA-35 were respectively used in experiments as substrates for methanogenic bacteria.

In studies with methanogens, 450 ml of Smith and Hungate medium (pH 7.0) with 1% sodium acetate, as a carbon source was taken in a 500 ml flask, flushed with nitrogen and inoculated with mixed cultures M-25 and M-35 as described above. The flask was fitted with a tube to collect the gas over a column of 0.1 N HCl. Flasks were incubated at 25°C and 35°C respectively and methane production was monitored. In some of the experiments, volatile fatty acids collected from digestion of cattle manure with C-25 and C-35 were used as substrates for methanogens. Methane production was also studied in the presence and absence of 1000 ppm IBA.

#### *Experiments with Rhodotorula spp*

The yeast belonging to *Rhodotorula* was isolated through standard soil enrichment techniques in Saboraud's medium containing 0.1% ammonium isobutyrate. The isolate with ability to grow on isobutyrate under anaerobic conditions was selected for the study. It was routinely maintained in stab culture in the same medium in the presence of 1000 ppm of IBA. In studies with methanogens, *Rhodotorula* was grown in Saboraud's broth containing 1000 ppm IBA for 48 h and the cells were centrifuged, washed in distilled water and resuspended at a cell concentration of 10 g wet weight per litre of Smith and Hungate medium containing 1000 ppm of IBA. The cells were preincubated for 7 days at 35°C before transferring them to the digester containing methanogens (M-35) growing on 1% sodium acetate.

#### *Analytical methods*

Cattle manure used as a substrate as well as the source of micro-organisms was analysed for protein, fat, crude fibre, total volatile matter (TVM), total solids (TS) according to the methods described in AOAC (1965). Volatile organic matter was measured by drying the sample in vacuum at 50°C and weighing before and after igniting it at 600°C. Loss in weight in grams was a measure of TVM.

Effluent from the digester was routinely monitored for reducing sugars (Bernfeld 1955), VFA (Rand *et al.* 1979) and total gas generated during digestion. Gas was monitored by GLC specially modified for the purpose. Gas samples were then injected in a chromosorb (80/100 mesh) packed column (6' x 1/8") to separate CH<sub>4</sub> and CO/CO<sub>2</sub>. The separated components were then passed over a nickel catalyst where CO/CO<sub>2</sub> were reduced to methane by molecular hydrogen. All components were detected as methane. Thus, there were two peaks, one corresponding to methane and other corresponding to CO/CO<sub>2</sub> detected as methane. The details of the equipment and method are described by Subramaniam (1980). Values are expressed as per cent of the two peak areas. The VFA from the effluent was recovered by steam distillation. After acidification and extraction with ether, it was analysed by GLC.

## Results

### *Composition of the cattle (buffalo) manure*

Buffalo manure is a major source of the substrate in a biogas plant. It also provides the necessary inputs for cellulolysis as well as methanogenesis for the digester. Principal carbon source in this substrate is cellulose (and some hemicellulose) which comprises about 37–38 % of the dry matter. Other possible substrates for anaerobic digestion such as lipids, proteins etc. are in minor amounts (table 1). Together they constitute about 81–84 % of volatile organic matter which is digestible. Fresh manure contains about  $10^4$  bacteria per g, most of which have their origin in rumen fluid and can survive biogas system only under strict anaerobic conditions. Among the substrates, cellulose is relatively more resistant to enzyme attack, as compared with other substrates such as proteins or lipids and consequently, under anaerobic conditions its degradation becomes a rate limiting step in biogas production.

**Table 1.** Composition of cattle (buffalo) manure.

Analysis	1	2
<i>Per cent total</i>		
Moisture	81	85
Total solids	19	15
<i>Per cent dry weight</i>		
<i>Components</i>		
Crude fibre	37	38
Protein	5.6	6.0
Fat	1.4	2.0
Ash	16.3	19.0
volatile organic matter	84	81
<i>Per g of solids</i>		
Total anaerobic bacteria	$1.2 \times 10^4$	$1.3 \times 10^4$

### *Profile of VFA produced at 25°C and 35°C in biogas digester*

Anaerobic fed batch digesters set up for screening cellulolytic cultures were used for analysis of VFA in their effluent streams after they were equilibrated for about 10 weeks at 25°C and 35°C. The data presented in table 2 shows that the VFA accumulated at 25°C contained about 87–88 % of its acids as IBA and IVA. The VFA at 35°C contained only 38 % as branched chain fatty acids. Butyric acid was the major component of VFA accounting for 45.7 % of the total. The digesters which were operated at temperatures less than 20°C showed very poor fermentation rates and practically no VFA accumulation (data not shown).

### *Production of VFA by purified C-25 and C-35 and its profile on different substrates*

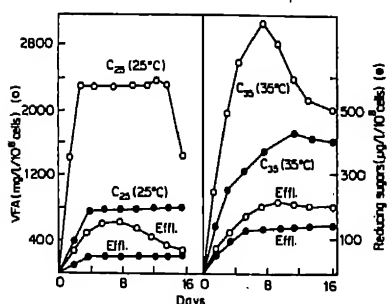
The conversion of cellulose and other organic constituents from the substrate to methane in a biogas digester involves a multitude of microbial systems and a study of

**Table 2.** Analysis of VFA from effluents and methane production in biogas digesters equilibrated at 25°C and 35°C.

VFA	Per cent of total fatty acids*	
	25°C	35°C
Acetate	Trace	Trace
Isopropionate	Trace	0.3
Propionate	2.5	0.6
Isobutyrate	32.6	16.7
Butyrate	3.6	45.7
Isovalerate	55.0	21.4
Valerate	1.1	3.9
Caproate	0.8	4.8
Unidentified	Trace	0.7
C <sub>7</sub> (?)	4.4	2.3
Caprylic	Trace	3.6
<i>Total gas</i>		
(L/Kg dung added/day)	0.13	0.38
<i>Methane</i>		
(L/Kg dung added/day)	0.03	0.20

\* Substrate for the biogas digester was cattle dung prepared as described in the text.

any one of them in isolation will not give a true picture of biochemical transformations taking place in the reactor. We have therefore chosen to study a mixed culture system which carry out important overall reactions leading to the formation of methane and CO<sub>2</sub>. Figure 1 shows the rates of formation of sugar and VFA from cattle manure by C-25 at 25°C and C-35 at 35°C. In this experiment, sterilised cattle manure was used as substrate. For comparison, the effluent from a standard operating KVIC digester with a capacity to produce gas at a rate of 0.1 m<sup>3</sup>/kg TVM/day was used as an inoculum. This digester has been operating at ambient temperatures and no effort was made to control temperatures. Fluctuations in temperature ranged from 26°C at night to 35°C during the day. Purified cultures showed almost three to four-fold improvement in fermentation rates when compared with the effluent culture from a standard KVIC biogas digester as measured by sugar and VFA content both at 25°C and 35°C.



**Figure 1.** Hydrolysis of cellulose and other carbohydrates in cattle manure waste (dung) to reducing sugar and VFA by mixed cultures C<sub>25</sub> at 25°C and C<sub>35</sub> at 35°C. For comparison, the effluent from a standard KVIC digester was used as inoculum. Reducing sugar (●); VFA (O).



Table 3. Composition of VFA formed with different substrates by C-25 and C-35.

Substrate*	Concentration g/L	Culture	Temp. (°C)	pH	Per cent of VFA					Total branched chain fatty acids (%)
					Acetic	Propionic	Butyric	Isobutyric	Isovaleric	
Cattle manure	80	C-25	25	5.4	1.5	0.5	8.6	90.0	< 0.5	90.0
			35	5.2	20.5	—	17.12	52.3	10.46	62.8
		C-35	25	6.4	4.0	9.1	61.0	7.8	18.1	25.9
			35	6.6	1.9	7.7	70.3	6.5	13.5	20.0
Glucose	80	C-25	25	6.5	8.2	4.0	85.5	0.6	2.0	2.6
			35	6.5	< 5.0	< 5.0	95.0	< 5.0	< 5.0	< 5.0
Filter paper	6	C-25	25	5.8	< 5.0	—	69.0	24.8	0.7	25.5

\* Experiments were carried out in batch culture (1 L) observing strict anaerobic conditions. The VFA was analysed after 10 days of incubation at respective temperatures.

Since the digesters operating at lower temperatures had shown accumulation of preferentially branched chain fatty acids with cattle manure as a substrate, a question may be asked whether the purified C-25 would also show a similar pattern, and if this property was true for other substrates as well. Results presented in table 3 clearly indicate that with cattle manure as substrate, mixed culture C-25 produced predominantly branched chain fatty acids at 25°C. The same culture grown at 35°C showed significant increase in acetic, butyric and isovaleric acids and reduction in IBA. A different VFA profile at a higher temperature by this culture is difficult to explain at present.

On the other hand, VFA produced by C-35 showed a pattern similar to that observed in digester operating at 35°C with butyric acid forming the predominant component, and its composition is not changed when the incubation temperature is 25°C. An interesting observation from the data presented in table 3 is the fact that the same culture C-25 switches over to produce straight chain fatty acids, predominantly butyric acid when glucose or filter paper is used as a substrate.

#### Effect of VFA-25 and VFA-35 on methane production by M-25 and M-35

Since the composition of VFA formed at different temperatures varies, how does it affect the methane formation? This was examined by collecting VFA from mixed cultures at C-25 at 25°C (VFA-25) and C-35 at 35°C (VFA-35), sterilising and using it as additive to methanogens growing on acetate at 25°C (M-25) and at 35°C (M-35). The data presented in table 4 shows a complete inhibition of methane formation from acetate by VFA-25 but not by VFA-35 suggesting a strong inhibitory effect of branched chain fatty acids, IBA and IVA present in VFA-25 on growth of aceticlastic methanogens.

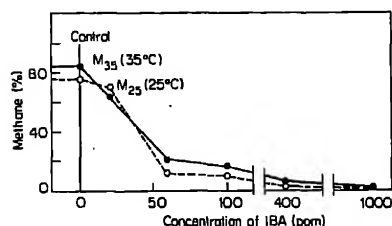
**Table 4.** Effect of VFA-25 and VFA-35 on methane formation from acetate by M-25 and M-35.

Culture	Substrates (g/l)	Temp. (°C)	pH	Time (days)	Total gas (l)	Methane	
						Volume (l)	%
	<i>Acetate</i>						
I-25	10	25	6.8	14	4.8	3.45	71.8
				12	4.3	3.14	73.0
I-35	10	35	6.9	14	4.1	3.15	76.8
				15	4.2	3.63	73.0
	<i>VFA + Acetate*</i>						
I-25	(VFA-25) 2.6	25	7.0	7	Nil	—	—
		35	6.8	7	Nil	—	—
	(VFA-35) 2.2	25	6.8	7	1.5	1.04	69.3
		35	6.8	7	2.2	1.50	67.8

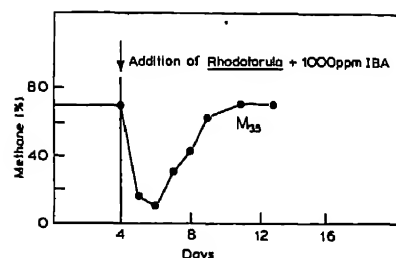
Concentration of acetate was 10 g/L. Concentration of VFA in effluent of the digester was 3.89 g/L for C-25 at 25°C and 2.2 g/L for C-35 at 35°C.

### Inhibition of methane formation by IBA and its reversal by *Rhodotorula*

Formation of methane from acetate was highly sensitive to inhibition by IBA even at concentrations as low as 50 ppm (figure 2). The inhibition was observed in the case of both the cultures M-25 and M-35 incubated at their respective temperatures of 25°C and 35°C. This inhibition was easily reversed by incorporating into the fermentation system *Rhodotorula* cells which were preincubated with 1000 ppm IBA presumably either by removing the inhibitor or converting it to a form easily metabolised by methanogens (figure 3). It is interesting to note that the methane production from acetate by methanogens was completely restored.



**Figure 2.** Inhibition of methane formation by IBA in M-25 and M-35 grown on acetate. Total incubation time—2 days. Temperature of incubation was 25°C for M-25 and 35°C for M-35. Average concentration of IBA in a digester operating at ambient temperature varies between 40–60 ppm.



**Figure 3.** Reversal of inhibition of methane production by IBA using yeast *Rhodotorula*. The culture M-35 was stabilised at 35°C on acetate as a carbon source for 4 days. The IBA (1000 ppm) was added along with the preadapted cells of *Rhodotorula*. Details of the Experiment are presented in the text.

### Discussion

The development of biogas generation system as an additional source of energy to meet the needs of rural areas of India has been given a high priority. Basic raw material for the biogas is cattle manure. The anaerobic digestion of organic constituents from the manure (table 1) to methane can be broadly considered to be due to the activity of three types of microbial systems *viz.* (i) fermentative microbes, which produce low molecular weight organic acids (VFA), (ii) acetogenic bacteria, and (iii) methanogens (Klass, 1984). We have presented evidence here to show that products of hydrolysis from the first two groups of micro-organisms have profound effects on methanogens which grow on acetate. About 72% of methane comes from aceticlastic methanogens (Mackie and Bryant, 1981; Fathepure, 1983; Klass, 1984). It would therefore be expected that inhibition of metabolic activity of these organisms will affect methane production. Fermentative microbes selected for lower temperature (~ 25°C) produce VFA rich in

branched chain fatty acids, particularly IBA and IVA. Accumulation of these acids inhibits growth of aceticlastic methanogens on acetate and their ability to produce methane. The inhibitory effects can be reversed by incorporating into the system another organism *Rhodotorula* which utilises IBA under anaerobic conditions.

These observations are significant because they explain the extreme sensitivity of biogas generation system to slight variation in temperature and its stabilisation by incorporation of other micro-organisms with a capacity to use branched chain fatty acids e.g. *Bacteroides ruminogen*, *Ruminococcus albus*, *Ruminococcus flavifaciens*, *Butyrivibrio fibrisolvens* etc. (Baldwin and Allison, 1983). These bacteria are normal constituents of rumen. Such an approach may also improve methane content of biogas since even in digester operating at 35°C, about 40% of the total VFA comprise of branched chain fatty acids (table 2). The yeast *Rhodotorula*, though capable of growing on IBA, cannot survive at low O/R potentials (– 350 mv) which are essential for the optimal activity of methanogens (Taylor, 1975). Selection of anaerobes for growth on branched chain fatty acids should therefore be one of the important approaches to overcoming the problem of inconsistent production of methane in biogas digester due to temperature shifts.

Origin of branched chain fatty acids with sterilised cattle manure as a substrate needs to be explained, since it does not happen to any significant extent when glucose is used as a substrate even with the mixed culture C-25 which normally accumulates them upto 69–90% of total VFA (table 3). When pure cellulose in the form of filter paper is used as a substrate, the same culture accumulates about 25% of the total VFA as branched chain fatty acids. This would suggest that at lower temperatures (25°C), polymers of glucose under anaerobic conditions have a tendency to be converted partially to branched chain fatty acids, a fact difficult to explain at present.

The practical importance of our observations is quite apparent because they provide an explanation as to why addition of reducing sugars like sugar cane molasses should improve methane production at lower temperatures in biogas digesters (Subramanian, 1977). On the basis of the results presented here, it is fair to assume that in molasses, the presence of invert sugar may favour the formation of straight chain fatty acids e.g. butyric acid rather than branched chain fatty acids, thereby improving the rates of methane formation.

It is interesting to note that in all cases, under conditions which reduce accumulation of branched chain fatty acids, there is a predominant shift to produce large amounts of butyric acid, a probable precursor to acetate formation during anaerobic digestion. There is a compelling evidence to suggest that acetogenic bacteria e.g. *Butyribacterium nethyilotrophicum* accumulates predominantly butyric acid and not acetate when grown on C<sub>1</sub> compounds such as methanol, CO<sub>2</sub> and formate (Kerby *et al.*, 1983). This apparently is derived from condensation of two acetyl-coenzyme A molecules to yield one butyrate. In methanogenesis, butyrate is a precursor for acetate, a natural substrate for aceticlastic methanogens.

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## Effect of retinol on the hemolysis and lipid peroxidation in vitamin E deficiency

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**Abstract.** A study on the effect of retinol *in vitro* on the hemolysis of vitamin E deficient rat red blood cells showed that retinol enhanced the lysis of the E deficient cells as compared to the lysis of normal cells. The lipid peroxidation present during hydrogen peroxide induced lysis of E deficient cells was however markedly inhibited in the presence of retinol without affecting the rate of lysis. In an actively peroxidising system of non-enzymatic lipid peroxidation of rat liver or brain homogenates and of brain lysosomes incubated with human erythrocytes, no lysis was obtained; incorporation of retinol in such systems resulted in lysis but no peroxidation. Hydrogen peroxide generating substances almost completely inhibited the lysis of normal human erythrocytes by retinol, but linoleic acid hydroperoxide and auto-oxidised liver or brain homogenates and ox-brain liposomes increased the lysis. It is concluded that vitamin E deficient erythrocyte hemolysis may be augmented by retinol, an anti-oxidant, having a lytic function without the peroxidation of stromal lipids.

**Keywords.** Normal human cells; vitamin E-deficient red blood cell; retinol lysis;  $H_2O_2$  generating agents; ox-brain liposomes; lipid peroxidation.

### Introduction

Among the well documented lytic actions of retinol, red cell hemolysis has been investigated extensively (Krishnamurthy and Kartha 1973; Lucy 1970). Mega dosing of retinol to rats has been shown to produce oxidative lysis of erythrocytes (Kartha and Krishnamurthy, 1978; Thomas George *et al.*, 1981). In an earlier communication we have shown that peroxidation of erythrocyte stromal lipids does not occur during retinol lysis of normal human erythrocytes (Krishnamurthy and Kartha, 1973). On the other hand we have adduced evidences for the involvement of reactive hydroxyl ( $OH^\cdot$ ) and  $H_2O_2$  radicals in retinol induced lysis (Krishnamurthy *et al.*, 1984). Along with normal human and rat cells, another type, *viz.* the  $\alpha$ -tocopherol deficient red blood cells which undergo oxidative damage and lysis during exposure to small amounts of  $H_2O_2$ , are also investigated in the present study. Both glucose-6-phosphate dehydrogenase deficient (Cohen and Hochstein, 1965) and vitamin E deficient erythrocytes (Cohen, 1975) respond to menadione during *in vivo* hemolysis. Certain other drugs induce hemolysis, *i.e.* phenylhydrazine and 8-hydroxy quinoline derivatives have been shown to be due to the toxicity of  $H_2O_2$  generated during the interaction of the drug with oxyhemoglobin (Cohen, 1975). Dialuric acid and glutathione have been shown to generate  $H_2O_2$  on *in vivo* incubation with vitamin E deficient red blood cells resulting in

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hemolysis and lipid peroxidation of cell membranes (Brownlee *et al.*, 1977; Goldberg and Stern, 1976). In the present study we are reporting the effect of retinol on two types of cells, normal and vitamin E deficient, in the light of the known antioxidant property, of retinol both *in vitro* and *in vivo* (Karthi and Krishnamurthy, 1978). Furthermore, we have studied the effect of  $H_2O_2$  generating agents and actively peroxidising system of non-enzymatic lipid peroxidation on the course of retinol lysis with both normal and vitamin E deficient erythrocytes. Our results demonstrate that the presence of retinol, markedly augmented vitamin E-deficient erythrocyte-lysis even in the absence of any oxidative challenge and the lysis was unaccompanied by stromal lipid peroxidation.

### Materials and methods

Synthetic retinol was obtained from Hoffman-la-Roche, Basle, Switzerland; other chemicals were obtained from SISCO Research laboratories, India.

Human blood, freshly drawn from healthy volunteers, was collected in ACD (acid-citrate-dextrose, National Institute of Health formula A of citric acid, sodium citrate and dextrose) and the separated cells were washed three times with saline phosphate (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl) and stored as 20% stock suspension in the same buffer at 4–6°C. Saline phosphate, pH 7.4 (of above composition) was used throughout the studies.

Hemolysis studies were done essentially following the procedure described earlier (Krishnamurthy and Karthi, 1973). Retinol (35  $\mu$ g) in 0.03 ml of aldehyde free ethanol was emulsified by adding 5 ml of saline phosphate buffer to which was added 5 ml of stock cell suspension. Additives were added either in ethanol or the buffer. Aliquots were withdrawn at specified time intervals for measuring hemolysis and thiobarbituric acid reactive products. Hemolysis with distilled water was taken as total hemolysis and the values were expressed as percentage of the total. Thiobarbituric acid reactive products are expressed in terms of absorbance at 535 nm. For oxidative hemolysis the system was the same as above but contained 220 mM  $H_2O_2$  (final concentration).

#### *Preparation of methemoglobin and carboxyhemoglobin cells*

Methemoglobin cells were prepared from normal human erythrocytes by treating them with sodium nitrite at a final concentration of 1% as described by Taylor and Hochstein (1977).

Cells containing carboxyhemoglobin were prepared by exposure to approximately 10% CO by injecting the gas into the air space of the flask through air-tight rubber caps as described by Kellogg and Fridovich (1975).

#### *Tissue homogenates*

Ten per cent homogenates of normal rat liver or brain were prepared in saline phosphate, pH 7.4, centrifuged at 10,000g for 10 min and the supernatants used.

#### *Preparation of ox-brain liposomes*

Ox-brain phospholipids were extracted and the liposomes were prepared in saline phosphate following the method of Guttridge (1977). Ox-brain (50 g) obtained from

local slaughter house was used. For preparation of liposomes, 10 mg of the phospholipid was dissolved in 0.2 ml of chloroform, the solvent was completely evaporated under vacuum and 10 ml of saline phosphate was added to the lipid film which was later brought into liposomal form by mechanical agitation and was then immediately used.

#### *$\alpha$ -Tocopherol deficient rats*

Weanling male albino rats (30) were fed a synthetic diet, consisting of vitamin free casein-20%, rice starch-50%, safflower oil-20%, cellulose 5% and salt mixture 4% and vitamin mixture except vitamin E. At the end of six weeks (100–120 g), blood was drawn from the tail vein and tested for oxidative (hydrogen peroxide) hemolysis. About 80% hemolysis and lipid peroxidation of the cells (TBA-index 0.13) were obtained within 60 min showing  $\alpha$ -tocopherol deficiency. Rats reared on such E deficient diet were sacrificed at the end of 6 weeks, bled by heart puncture while under light ether anaesthesia, into heparinised syringes. Liver  $\alpha$ -tocopherol content was determined following the procedure of Bieri *et al.* (1961) at the end of the feeding experiments and it was less than 1  $\mu\text{g}/5\text{ g}$ , confirming that the diet was able to produce adequate  $\alpha$ -tocopherol deficiency.

#### Results and discussion

The results presented in table 1 shows that both normal and vitamin E deficient erythrocytes were readily lysed by retinol but unaccompanied by membrane lipid peroxidation. Lysis of vitamin E deficient erythrocytes in the presence and absence of  $\text{H}_2\text{O}_2$  was augmented by retinol. Oxidative hemolysis of  $\alpha$ -tocopherol deficient erythrocytes was associated with membrane lipid peroxidation confirming previous reports (Brownlee *et al.*, 1977; Krishnamurthy and Bieri, 1961). The presence of 35  $\mu\text{g}$  of retinol in the oxidative lysis system of vitamin E deficient erythrocytes enhanced the lysis from 65% to 100% but inhibited the thiobarbituric acid reactive material by 38%.

Table 1. Retinol induced lysis of normal and  $\alpha$ -tocopherol deficient rat erythrocytes.

	Percentage hemolysis				
	Time in min				
	0	15	30	45	60
Normal rat cells	0	0	2	5	5(0.00)**
Normal rat cells + retinol (35 $\mu\text{g}$ )	4	30	52	68	86(0.00)**
$\alpha$ -Tocopherol deficient cells	2	5	5	7	8(0.00)
$\alpha$ -Tocopherol deficient cells + retinol (35 $\mu\text{g}$ )	2	40	68	95	100(0.00)
$\alpha$ -Tocopherol deficient cells + $\text{H}_2\text{O}_2^*$	4	18	31	44	65(0.13)
$\alpha$ -Tocopherol deficient cells + $\text{H}_2\text{O}_2$ + retinol (35 $\mu\text{g}$ )	5	53	80	100	100(0.08)

\*  $\text{H}_2\text{O}_2$ —final concentration 220 mM.

\*\* Values in parenthesis indicate the increase in absorbance at 535 nm over 60 min.

Retinol was emulsified using 0.03 ml ethanol and this had no effect on the hemolysis.



Retinol at concentrations of 70  $\mu\text{g}$  and 100  $\mu\text{g}$  produced 100% lysis at the end of 15 and 0 min but inhibited peroxidation by 69 and 85% respectively (data not given).

In the case of normal cells also 70  $\mu\text{g}$  and 100  $\mu\text{g}$  of retinol produced complete lysis at the end of 15 and 0 min. Thus retinol, hemolytic agent by itself could augment both the autohemolysis and oxidative lysis of  $\alpha$ -tocopherol deficient cells to a marked degree more than observed from normal cells. Further, retinol inhibited the stromal lipid peroxidation of vitamin E deficient cells.

Cohen and Hochstein (1965) have shown that hemolytic drugs like phenylhydrazine, menadione, 1,2-naphthaquinone and hydroquinone could liberate a flux of  $\text{H}_2\text{O}_2$  intracellularly with simultaneous oxidation of oxyhemoglobin to methemoglobin. Results in table 2 show the effect of such drugs on retinol lysis of normal human erythrocytes. The hemolysis was almost completely inhibited by the hemolytic drugs. In separate experiments, human erythrocytes containing methemoglobin and carboxyhemoglobin prepared as described under 'materials and methods' were subjected to retinol induced lysis. Only 10 and 16% hemolysis were observed in methemoglobin and carboxyhemoglobin cells as against 91% for normal human red cells (data not given). These results could mean that the presence of oxyhemoglobin is obligatory for the retinol induced lysis.

**Table 2.** Effect of  $\text{H}_2\text{O}_2$  generating agents on retinol induced hemolysis of normal human erythrocytes

Additives	Concentration (mM)	Percentage hemolysis			
		Time in min			
		0	15	30	60
None	—	4	37	60	95
Phenylhydrazine	1.5	0	0	0	3
Menadione	1.5	0	0	2	6
1,2-naphtha quinone-4-sulphonic acid	1.5	0	2	5	5
Hydroquinone	2.0	0	12	16	18

Values are mean of six separate sets of experiments.

McCay *et al.* (1972) have shown that normal erythrocytes were hemolysed when incubated in an actively peroxidising system of rat liver microsomes, ADP,  $\text{Fe}^{2+}$  and NADPH and have concluded that the hemolysis was a result of red cell membrane damage due to the enzymatic lipid peroxidation of the microsomes. The effect of non-enzymatic peroxidative system on retinol induced lysis of normal human erythrocytes was studied and results are presented in table 3. Three non-enzymatic peroxidising systems were studied—rat liver, brain homogenates and ox-brain liposomes in the presence of  $\text{Fe}^{2+}$ . These systems by themselves produced no hemolysis of normal human erythrocytes. The presence of retinol along with the peroxidising system resulted in lysis of human erythrocytes. The degree of lysis was the same irrespective of the presence or absence of the peroxidising system. However, retinol markedly inhibited the lipid peroxidation in all the three systems over a period of 60 min.

Preincubation of rat liver or brain homogenates and liposomes with  $\text{Fe}^{2+}$  produced

Table 3. Lipid peroxidation and retinol induced hemolysis of normal erythrocytes

	Percentage hemolysis			TBA-index at 535 nm	
	Time in min				
	0	30	60	0	60
cells <sup>a</sup> + retinol <sup>b</sup>	5	60	96	0.02	0.02
ml brain homogenate + cells	0	0	0	0.06	0.80
ml brain homogenate + cells + retinol	2	60	96	0.06	0.07
ml preincubated brain homogenate + cells + retinol	5	86	100	0.08	0.81
ml liver homogenate + cells	0	0	0	0.03	0.54
ml liver homogenate + cells + retinol	2	65	100	0.05	0.05
ml preincubated liver homogenate + cells + retinol	4	82	100	0.55	0.55
ox-brain liposomes <sup>c</sup> + cells	0	0	0	0.00	0.02
ox-brain liposomes <sup>c</sup> + Fe <sup>2+</sup> + cells	0	0	0	0.04	0.29
ox-brain liposomes + Fe <sup>2+</sup> + cells + retinol	2	60	98	0.00	0.00
ox-brain liposomes preincubated with Fe <sup>2+</sup> + cells + retinol	10	98	98	0.30	0.29
LAHPO <sup>e</sup> + cells + retinol	8	100	100	0.32	0.30

One ml of 20% cell suspension of human erythrocytes in saline phosphate.

35 µg of retinol in 0.03 ml ethanol.

Liposomes were prepared with 10 mg of ox-brain phospholipid in 10 ml saline phosphate. One ml of the suspension was used.

Ox-brain liposomes were incubated with Fe<sup>2+</sup> (0.4 mM) for 30 min. An aliquot was withdrawn and the absorbance at 535 nm was determined.

LAHPO prepared from pure linoleic acid and equivalent to 100 nmol of MDA were incubated in the system.

appreciable amount of thiobarbituric acid reactive material. Addition of these to the hemolytic system enhanced the retinol induced lysis pointing out that preformed hydroperoxides can increase the lysis by retinol. Further, retinol cannot act as an inhibitor of already formed peroxides. This is further supported by the results of additions of linoleic acid hydroperoxides to the hemolysing system which resulted in a complete lysis of cells over a period of 30 min as against only 60% lysis of normal red-cells by retinol.

The lysis of  $\alpha$ -tocopherol deficient cells is shown to be peroxidation dependent (Krishnamurthy and Bieri, 1961). According to Brownlee *et al.* (1977) in the E deficient cells undergoing oxidative lysis the stromal lipid peroxidation is initiated by singlet oxygen radical while the lysis is attributed to hydroxyl radical. Retinol has been shown to inhibit mixed function oxidase by its ability to quench singlet oxygen (Hill and Shih, 1974) and the results of the present study may be taken as confirming the ability of retinol to quench singlet oxygen and thus preventing lipid peroxidation but not hydroxyl radicals, which is probably the cause of lysis, as reported by us (Krishnamurthy *et al.*, 1984) in an earlier study.

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## Effect of 6-Aminonicotinamide on the activity of hexokinase and lactate dehydrogenase isoenzymes in regions of the rat brain

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**Abstract.** Changes in the activity of hexokinase and lactate dehydrogenase isoenzymes in the three brain regions and heart were studied in the 6-Aminonicotinamide-treated rats. Drug administration decreased the particulate hexokinase and lactate dehydrogenase activity, but increased the soluble hexokinase.

**Keywords.** Hexokinase; lactate dehydrogenase; isoenzymes; brain; glycolysis.

### Introduction

In animal tissues, nicotinic acid is mostly found in the nicotinamide moiety of the two coenzymes,  $\text{NAD}^+$  and  $\text{NADP}^+$ , which are involved in the anaerobic breakdown of glucose, citric acid cycle, and amination of glutamic acid (Sternberg and Phillips, 1959). 6-Aminonicotinamide (6-AN) an antimetabolite of nicotinamide when administered to animals, forms an inactive analogue of  $\text{NADP}$  causing effects mainly in the nervous system (Herken, 1968b; Herken *et al.*, 1974).

Investigations on the effect of 6-AN on the pathways of glucose utilization in brain (Lange *et al.*, 1970; Hothersall *et al.*, 1981) showed that the compound primarily influences the pentose phosphate pathway, leading to the accumulation of 6-phosphogluconate which in turn decreases glycolytic flux by inhibiting phosphoglucomutase.

The brain largely depends on glucose for its energy metabolism. Earlier reports on 6-AN-treated rats showed significant changes in the levels of glucose, glucose-6-phosphate, lactate, ATP etc. (Lange *et al.*, 1970; Hothersall *et al.*, 1981). The purpose of the present study was to understand further the effects of 6-AN on glucose metabolizing pathways in the brain. The key position of hexokinase and lactate dehydrogenase in the cellular metabolism of glucose makes the study of these enzymes of great interest. In the present work, isoenzymes of these enzymes were studied. Heart tissue was also studied for comparison.

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Abbreviations used: 6-AN, 6-Aminonicotinamide; TPF, total particulate fraction.

## Materials and methods

Albino rats of Wistar strain weighing between 180–200 g were used. 6-AN dissolved in 0.9% saline was injected (*i.p.* 35 mg/kg body wt.) to each rat. The animals were killed at specified time intervals after injection. The control rats were injected 0.9% saline and the enzyme activities were determined at the same time post injection.

### *Preparation of homogenates*

Rats were killed by cervical dislocation and the cerebral hemispheres, cerebellum, brain stem and the heart were excised immediately and weighed. Tissue homogenates (1:10) were prepared in isotonic sucrose medium as described earlier (Kaur *et al.*, 1983). The extracts were centrifuged at 12,000 *g* for 40 min and the supernatant was used as the soluble fraction(s). The pellet was washed once and resuspended in the same medium to obtain total particulate fraction (TPF).

### Enzyme assay

Hexokinase activity was estimated essentially according to the method of Sharma *et al.* (1963) as modified by Gumaa and McLean (1972). The TPF was treated with Triton-X-100 (final concentration 0.5%) in the cold for 30–60 min to liberate latent and bound enzymes. For the estimation of the activity of isoenzymes, aliquots of each fraction were heated at 45°C for 1 h (Grossbard and Schmike, 1966). This process destroyed hexokinase Type II completely and the subtraction of the activity of the heated fraction from the total gave the activity of Type II isoenzyme. Lactate dehydrogenase activity was assayed by the method of Bergmeyer and Bernt (1974) and gel electrophoresis was done by the method of Ornstein (1964). One unit of activity for hexokinase and lactate dehydrogenase was defined as the amount required to form one  $\mu\text{mol}$  of NADPH or  $\text{NAD}^+$ , respectively per min at 25°C.

## Results

### *Changes in hexokinase activity in the brain regions and the heart*

The total hexokinase in TPF from the cerebral hemispheres showed a significant decrease in activity after 8 and 16 h of drug (6-AN) administration, whereas, the soluble hexokinase showed an increase. Hexokinase Type I isoenzyme in TPF decreased ( $P < 0.01$ ) and this was accompanied by an increase in the soluble fraction enzyme ( $P < 0.01$ ) at 16 h. A significant decrease in the activity of hexokinase Type II in TPF was observed at 8 and 16 h ( $P < 0.02$  and  $P < 0.05$  respectively). The hexokinase associated with soluble fraction increased at 16 h after drug administration. The results are presented in table 1 and figure 1A.

The activity of the total hexokinase from the cerebellum in TPF did not change after the drug administration. However, the soluble enzyme showed a significant increase at 16 h. Hexokinase Type I in both the subcellular fractions did not change. The activity of hexokinase Type II was 67–80% of the control levels at 2, 8 and 16 h of drug treatment.

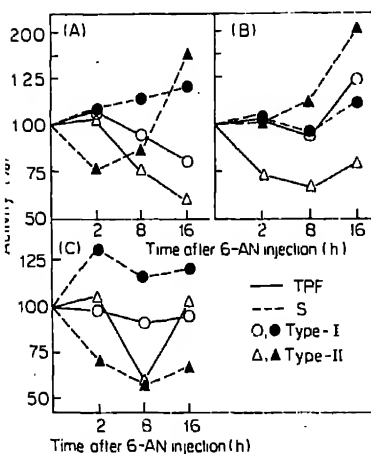
**Table 1.** Effect of 6-AN on total hexokinase from TPF and soluble fraction in different regions of the brain and the heart.

Region	Fraction	Control	Time after 6-AN injection (h)		
			2	8 units/g	16
Cerebral hemispheres	TPF	6.8 ± 0.15	7.24 ± 0.07	6.1 ± 0.08 <sup>c</sup>	5.1 ± 0.2 <sup>e</sup>
	S	3.0 ± 0.11	3.1 ± 0.01	3.1 ± 0.1	3.8 ± 0.14 <sup>d</sup>
Cerebellum	TPF	3.6 ± 0.27	3.6 ± 0.03	3.1 ± 0.04	4.0 ± 0.17
	S	2.8 ± 0.15	2.9 ± 0.11	2.8 ± 0.14	3.6 ± 0.11 <sup>c</sup>
Brain stem	TPF	2.8 ± 0.16	3.0 ± 0.08	2.4 ± 0.23	2.8 ± 0.09
	S	2.5 ± 0.22	3.0 ± 0.03	2.6 ± 0.14	2.7 ± 0.13
Heart	TPF	1.6 ± 0.29	1.3 ± 0.02	0.96 ± 0.06	1.9 ± 0.17
	S	3.4 ± 0.17	2.7 ± 0.08 <sup>b</sup>	3.7 ± 0.25	4.0 ± 0.16 <sup>a</sup>

Each value is a mean ± SEM of four or more experiments, done in triplicates.

TPF, Total Particulate Fraction; S, Soluble fraction.

<sup>a</sup>  $p < 0.05$ ; <sup>b</sup>  $p < 0.02$ ; <sup>c</sup>  $p < 0.01$ ; <sup>d</sup>  $p < 0.002$  and <sup>e</sup>  $p < 0.001$ .



**Figure 1.** Per cent activities of hexokinase Type I and Type II isoenzymes in TPF and soluble fraction from (A) cerebral hemispheres (B) cerebellum and (C) brain stem, after 6-AN treatment. Absolute activities of hexokinase Type I and Type II isoenzymes in TPF were  $5.5 \pm 0.2$  and  $1.3 \pm 0.01$ ;  $2.8 \pm 0.3$  and  $0.7 \pm 0.1$  and  $2.3 \pm 0.2$  and  $0.5 \pm 0.04$  and in soluble fraction were  $2.3 \pm 0.1$  and  $0.72 \pm 0.05$ ;  $2.3 \pm 0.1$  and  $0.5 \pm 0.06$  and  $1.9 \pm 0.2$  and  $0.65 \pm 0.08$  from the cerebral hemispheres, cerebellum and the brain stem respectively. Each value is a mean ± SEM of 4 or more experiments, done in triplicates.

The changes however were not significant. Hexokinase Type II in soluble fraction increased at 16 h ( $P < 0.02$ ) after injection. The results are presented in table 1 and figure 1B.

Total hexokinase activity in the TPF and soluble fraction and Type I isoenzyme in the TPF from the brain stem showed only marginal changes at all the three intervals of time after drug administration. Soluble fraction hexokinase Type I increased to 132% at 16 h. After 8 h, hexokinase Type II activity in both the subcellular fractions decreased significantly ( $P < 0.01$  and  $P < 0.05$  respectively). The results are presented in table 1 and figure 1C.

The total hexokinase activity in TPF from the heart tissue was significantly decreased after 8 h, whereas the soluble fraction enzyme showed a decrease at 2 h

followed by a gradual rise in the activity upto 16 h. A significant decrease in hexokinase Type I from TPF occurred at 8 h ( $P < 0.02$ ) with a subsequent increase at 16 h ( $P < 0.01$ ). Hexokinase Type I in the soluble fraction showed increase at 8 and 16 h ( $P < 0.02$  and  $P < 0.01$  respectively). Type II TPF hexokinase increased at 2 and 16 h ( $P < 0.05$  and  $P < 0.02$  respectively). Soluble Type II showed a decrease at 2 h ( $P < 0.01$ ). The results are presented in table 1 and figure 2.

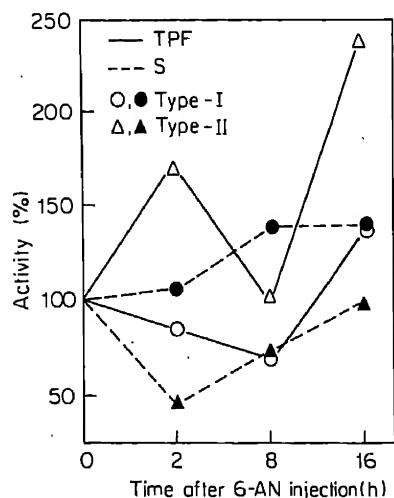


Figure 2. Activity of hexokinase Type I and Type II isoenzymes in TPF and soluble fraction from the heart following 6-AN treatment. Absolute activities of hexokinase Type I and Type II are  $0.95 \pm 0.06$  and  $0.3 \pm 0.04$  and  $1.8 \pm 0.1$  and  $1.6 \pm 0.1$  and in TPF and soluble fraction respectively.

#### Changes in lactate dehydrogenase from the brain regions and the heart

Lactate dehydrogenase activity in the cerebral hemispheres decreased significantly at 8 and 16 h after drug administration. The enzyme activity in the cerebellum and the brain stem showed minimal changes after 6-AN injection. In the heart, lactate dehydrogenase activity showed a significant increase at 16 h. The results are presented in table 2.

The gel electrophoretic pattern of the isoenzymic forms of lactate dehydrogenase are presented in figure 3. A significant decrease in the Type III and IV isoenzymes in the

Table 2. Effect of 6-AN on lactate dehydrogenase in different regions of the rat brain and the heart.

Region	Control	Time after 6-AN injection (h)		
		2	8 units/g	16
Cerebral hemispheres	$27.7 \pm 1.08$	$25.2 \pm 0.14$	$18.3 \pm 0.6^c$	$24.2 \pm 0.44^b$
Cerebellum	$19.8 \pm 1.6$	$20.2 \pm 0.14$	$18.1 \pm 0.44$	$22.9 \pm 1.3$
Brain stem	$16.2 \pm 1.4$	$13.1 \pm 0.33$	$12.9 \pm 0.49$	$15.5 \pm 0.54$
Heart	$109.6 \pm 5.1$	$104.2 \pm 5.3$	$115.8 \pm 2.8$	$129.6 \pm 6.5^a$

Each value is a mean  $\pm$  SEM of four or more experiments, done in triplicates.

<sup>a</sup>  $p < 0.05$ ; <sup>b</sup>  $p < 0.02$  and <sup>c</sup>  $p < 0.001$ .

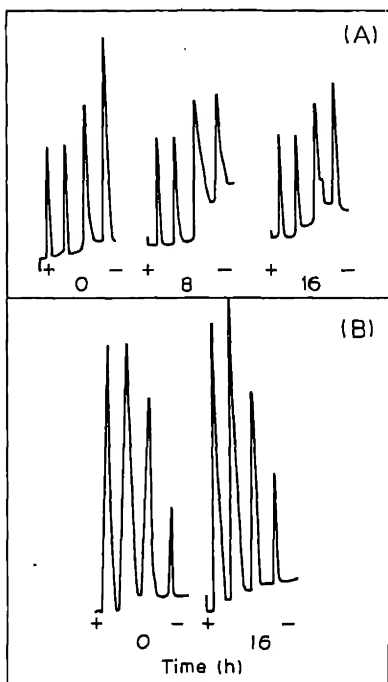


Figure 3. Densitometric tracings of the polyacrylamide gel electrophoresis of lactate dehydrogenase isoenzymes in (A) cerebral hemispheres and (B) heart from 6-AN treated rats. (C) Control at 8 and 16 h after drug treatment in the cerebral hemispheres and the heart.

cerebral hemispheres was observed at 8 and 16 h after 6-AN treatment, whereas, in the heart significant increase in the amount of all the four isoenzymes was observed.

## Discussion

Significant neurological symptoms have been reported after administration of 6-AN to rats. Herken *et al.* (1974) showed that after 6–8 h of drug administration, the antimetabolite became fixed cellularly in the brain, which was unable to act as a hydrogen carrier in oxidation–reduction reactions, utilizing NADP. In the present experiments, significant effects of 6-AN on the enzyme activities were seen after 8 h of drug treatment.

A significant decrease in the particulate hexokinase was observed, with an increase in the soluble form but with no significant change in the total hexokinase. Glucose-6-phosphate level in the brain after 6-AN treatment increases considerably as compared with controls (Herken *et al.*, 1974; Lange *et al.*, 1970). From the results of *in vitro* and *in vivo* experiments, it has been suggested that a soluble-bound hexokinase equilibrium is very much sensitive to the metabolite control, in particular to glucose-6-phosphate (Wilson, 1980). Copley and Fromm (1967) reported that bound hexokinase has a high  $K_i$  for glucose-6-phosphate and a lower  $K_m$  for ATP as compared to the soluble form, suggesting that the bound form of hexokinase in the brain is the more active form. The present results suggest that the more active form (bound) of hexokinase in the rat brain



is converted to a less active form (soluble) under conditions of reduced cerebral energy metabolism.

The decrease in the hexokinase activity in the heart tissue may be due to the inhibition of insulin release by 6-AN. Ammon and Steinke (1972) showed that in pancreatic islets, decreased activity of the pentose phosphate pathway and reduced NADPH level induced by 6-AN, are associated with a reduced insulin response to glucose.

6-AN significantly decreased the lactate dehydrogenase activity in the brain. The evidence for an inhibition in the lower segment of the glycolytic pathway in 6-AN-treated rats is evident from the changes in the metabolite profile of this pathway. Lange et al. (1970) and Hotherhall et al. (1981) reported a decline in pyruvate and lactate levels of 6-AN treated rats. The enzyme activity decrease observed in the present work may be due to substrate deficiency.

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## Interactions of the channel forming peptide alamethicin with artificial and natural membranes

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**Abstract.** Alamethicin and related  $\alpha$ -aminoisobutyric acid peptides form transmembrane channels across lipid bilayers. This article briefly reviews studies on the effect of alamethicin on lipid phase transitions in lipid bilayers and on mitochondrial oxidative phosphorylation. Fluorescence polarization studies, employing 1,6-diphenyl-1,3,5-hexatriene as a probe, suggest that alamethicin fluidizes lipid bilayers below the phase transition temperature, but has little effect above the gel-liquid crystal transition point. Alamethicin is shown to function as an uncoupler of oxidative phosphorylation in rat liver mitochondria. The influence of alamethicin on mitochondrial respiration is modulated by the phosphate ion concentration in the medium. Classical uncoupling activity is evident at low phosphate levels while inhibitory effects set in at higher phosphate concentrations. Time-dependent changes in respiration rates following peptide addition are rationalized in terms of alamethicin interactions with mitochondrial membrane components.

**Keywords.** Alamethicin; membrane channels; mitochondrial uncouplers; mitochondrial inhibitors; peptide-lipid interactions; membranes.

### Introduction

Alamethicin and related  $\alpha$ -aminoisobutyric acid (Aib) containing peptides of microbial origin, form voltage dependent channels across lipid bilayer membranes (Mathew and Balaram, 1983a, 1984; Jung *et al.*, 1981; Mueller and Rudin, 1968; Mueller, 1976; Hall, 1978). These systems provide an excellent model for investigating the molecular events involved in the formation and gating of membrane channels. Over the past few years considerable effort has been devoted towards establishing the structural characteristics of these channel forming peptides (Nagaraj and Balaram, 1981; Prasad and Balaram, 1984; Toniolo *et al.*, 1983; Smith *et al.*, 1981; Fox and Richards, 1982; Banerjee *et al.*, 1983; Schmitt *et al.*, 1982). Figure 1 lists a few representative sequences for this class of peptides. The general consensus of these studies has favoured an overall helical conformation for these polypeptides, with channels being formed by aggregation of peptides in the lipid bilayer, generating supramolecular structures having a central aqueous core (figure 2) (Iqbal and Balaram, 1981; Fox and Richards, 1982).

An important feature of  $3_{10}$  or  $\alpha$ -helical peptide structures is the existence of a macrodipole moment along the helix axis, with the amino and carboxy termini serving

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Abbreviations used: Aib,  $\alpha$ -Aminoisobutyric; DPH, 1,6-diphenyl-1,3,5-hexatriene; P, polarization; DPPC, dipalmitoylphosphatidyl choline; DMPC, dimyristoylphosphatidyl choline;  $P_i$ , phosphate ions; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

Alamethicin I (II) : Ac-Aib-Pro-Aib-Ala-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro  
 (Aib)  
 -Val-Aib-Aib-Glu-Gln-Phol

Suzukacillin A (B) : Ac-Aib-Ala-Aib-Ala-Aib-Gln-Aib-Aib-Aib-Gly-Leu-Aib-Pro-Val  
 (Iva) (Aib) (Leu)  
 -Aib-Iva-Glu-Gln-Phol  
 (Aib) (Gln)

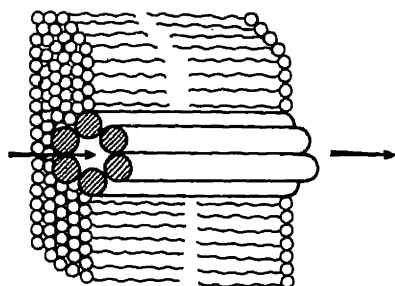
Trichotoxin A 40/50 : Ac-Aib-Gly-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Ala-Aib-Aib-Pro-Leu  
 (Ala) (Ala)  
 -Aib-Iva-Glu-Valal  
 (Aib) (Gln)

Emerimicin III (IV) : Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp  
 -Ala-Phol  
 (Aib)

Zervamicin II A : Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp  
 -Aib-Pro-Phol

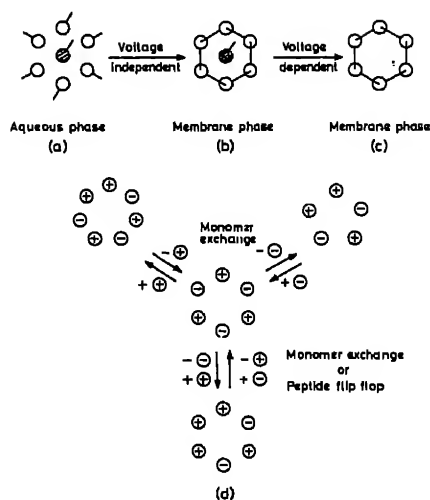
Paracelsin A : Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Aib-Aib  
 -Pro-Val-Aib-Aib-Gln-Gln-Phol

**Figure 1.** Representative sequences of Aib containing membrane channel forming polypeptides. All the naturally occurring peptides in this class are mixtures of very closely related polypeptides. A more complete listing of sequences may be found in Mathew and Balaram (1984). A discussion of separation and sequencing studies, with a critical analysis of heterogeneity of sequences is given by Bruckner and Przybylski (1984).



**Figure 2.** Schematic representation of transmembrane channel formation by lipid phase aggregation of helical, rodlike peptide structures.

as the positive and negative ends of the dipole, respectively (Wada, 1976; Hol *et al.*, 1981). In the case of alamethicin and the related peptide trichotoxin, dipole moments of 40–75 D have been estimated experimentally in solvents like octanol and dioxane (Yantorno *et al.*, 1982; Schwarz and Savko, 1982a,b; Schwarz *et al.*, 1983). Dipole-dipole interactions may therefore play an important role in stabilizing aggregates of membrane spanning peptides in the lipid phase (Mathew and Balaram, 1983a,b, 1984). The conductivity characteristics of channels may then be modulated by changing aggregate size and also by altering transmembrane orientations of peptide monomers in the aggregate, thus changing the net dipole moment of the channel. Two recent models, which attempt to rationalise available experimental observations on potential dependent channel formation by alamethicin and data on pore state fluctuations, have ascribed a key role to helix dipole interactions (Mathew and Balaram, 1983b; Balaram, 1983; Boheim *et al.*, 1983; Jung *et al.*, 1983). Figure 3 summarises the main features of a model for alamethicin channels proposed from this laboratory. It is clear that both peptide-peptide and peptide-lipid interactions will determine channel properties. For



**Figure 3.** Proposed model for voltage dependent channel formation by alamethicin. (a) Aggregate of peptide helices in the aqueous phase. The projection represents a polar sidechain, presumably Gln (7). (b) Membrane phase aggregate formed by insertion of peptide aggregate into the bilayer and rotation about the helix axis of each molecule to form intermolecular hydrogen bonds. A central peptide molecule (core piece) blocks the channel. (c) Voltage dependent extrusion of the core piece which would be a macrodipole. This is facilitated by lack of intermolecular hydrogen bonds to its neighbours. (d) Model for pore state transitions in which net dipole state or aggregation number changes. This occurs either by monomer exchange or peptide flip-flop across the bilayer (Mathew and Balaram, 1983b; Balaram, 1983).

example, pore state lifetimes could be determined by membrane lipid 'fluidity'; a more mobile lipid phase permitting facile peptide diffusion, leading to rapid formation and breakdown of functional aggregates. The effects of alamethicin and related amphipathic, helical peptides on natural membranes may be influenced by dipole-dipole interactions of the peptide with transmembrane segments of integral proteins.

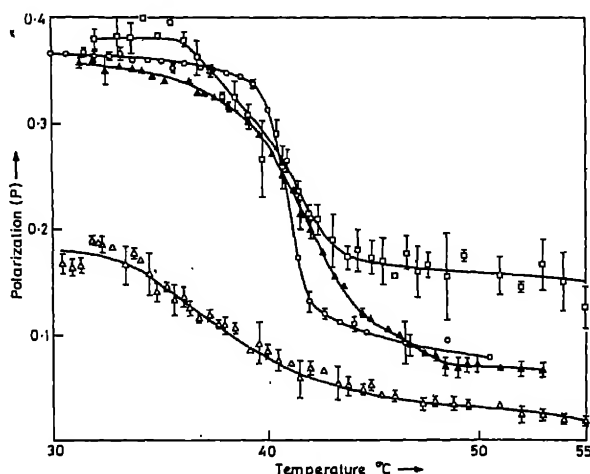
Detailed accounts of the structural characteristics of alamethicin and its synthetic fragments (Nagaraj and Balaram, 1981; Prasad and Balaram, 1984; Jung *et al.*, 1981; Fox and Richards 1982) and discussions of membrane modifying properties are available (Mathew and Balaram 1983a, 1984). There have been relatively few reports on the effects of alamethicin incorporation on the structural properties of artificial and natural membranes (Chapman *et al.*, 1969; Hauser *et al.*, 1970; Finer *et al.*, 1969; Lau and Chan, 1974, 1975, 1976; McIntosh *et al.*, 1982; Irmischer and Jung, 1977; Bessler *et al.*, 1979), in contrast to the large volume of electrochemical data on black lipid membrane systems. The observations that alamethicin pores result in the unmasking of inner surface membrane enzymes in diverse systems (Jones *et al.*, 1977, 1980; Lad and White, 1979; Besch *et al.*, 1977; Herman *et al.*, 1980; Misselwitz *et al.*, 1979; Bonnafant *et al.*, 1982; Monneron and d'Alayer, 1980; Bessler *et al.*, 1979), suggest that large channels can form in natural membranes. These, in turn, permit transmembrane passage of bulky, charged substrates. The observation that alamethicin (Takaishi *et al.*, 1980; Mathew *et al.*, 1981a) and its synthetic fragments (Mathew *et al.*, 1981a,b, 1982)

uncouple mitochondrial oxidative phosphorylation, has been interpreted in terms of effective dissipation of  $H^+$  gradients across the inner mitochondrial membrane by alamethicin channels (Mathew *et al.*, 1981a, 1982). This article briefly reviews recent results from this laboratory on the effects of alamethicin on artificial lipid bilayers and oxidative phosphorylation in mitochondria.

### Peptide-lipid interactions

Fluorescence polarization studies, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe, can be employed to monitor the physical state of lipid bilayers. The gel-liquid crystal phase transition, is accompanied by a substantial increase in the disorder of the hydrocarbon chains of lipid molecules, resulting in increased rotational and translational mobilities of dissolved spectroscopic probes. In the case of DPH incorporated into lipid bilayers, there is a large increase in rotational reorientation rates above the phase transition, leading to a significant fall in the value of the fluorescence polarization (P). DPH has therefore, been extensively employed as a probe of the microviscosity of artificial and natural membranes (Shinitzky and Barenholz, 1978).

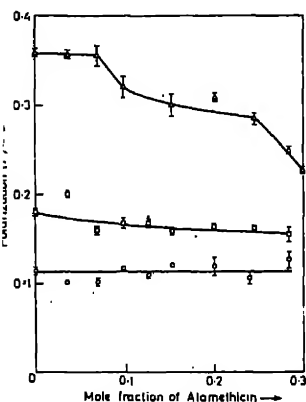
Figure 4 shows the results of experiments designed to monitor the effects of peptide



**Figure 4.** Effect of peptides on the phase transition of DL- $\alpha$ -DPPC liposomes monitored using DPH fluorescence polarization  $P$  as a probe. (O), DPPC; (□), DPPC + suzukacillin fragment 16 G; (Δ), DPPC + alamethicin fragment Z-1-17-OH; (Δ), DPPC + alamethicin. Peptide: lipid mol ratio is 1:2.3. DPPC (1.09  $\mu$ mol) and peptide (0.46  $\mu$ mol) were taken in  $CHCl_3$ - $CH_3OH$  (2:1 v/v) and the solvent evaporated in a stream of nitrogen. The film was dried under vacuum, 2 ml 5 mM HEPES-100 mM NaCl, pH 7.0 was added and the solution heated to 45°C and mixed vigorously on a vortex mixer. The suspension was sonicated until it became optically clear (Branson B-12 sonifier, output power 40 W). The solution was centrifuged to remove particulate matter. 1 ml of 2  $\mu$ M DPH in the same buffer was added to 1 ml of the liposome solution. The solutions were incubated at 45°C for 2 h to permit DPH incorporation. Polarization values were measured on a Perkin-Elmer MPF-44 A fluorescence spectrometer equipped with a polarization accessory.  $\lambda_{ex} = 360$  nm,  $\lambda_{em} = 430$  nm. Temperature control was accomplished using a thermostatted cell assembly and a controlled temperature water circulator. Polarization values are averages of 4 determinations.

incorporation on the thermal phase transition of dipalmitoylphosphatidyl choline (DPPC) liposomes. In the absence of peptide DPPC shows a sharp transition centered at 41°C, with P values of 0.36–0.37 below the transition and values of ~0.08 above the transition temperature. Incorporation of 30 mole per cent of alamethicin, dramatically perturbs the bilayer, resulting in a large drop in the P value even at 30°C. On heating the lipid-peptide complexes, a broad transition is observed, with very low P values (~0.03) at higher temperatures. Alamethicin thus appears to "fluidize" the gel phase of DPPC liposomes. A 17-residue synthetic fragment of alamethicin (benzyloxycarbonyl-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OH, Z-1-17-OH) and a 16-residue suzukacillin fragment (Boc-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe, 16G) were also examined. Figure 4 demonstrates that both these peptides have a much less pronounced effect on DPPC bilayers, as compared to alamethicin. In both cases there is a detectable broadening of the phase transition. It is of interest to note that the limiting P value at high temperature is significantly greater in the case of the neutral peptide 16 G, as compared to DPPC alone or in the presence of alamethicin or Z-1-17-OH. This may imply that 16 G forms a relatively more tightly packed peptide lipid complex above the lipid phase transition temperature. Both the 16 and 17 residue peptides have been shown to form channels which transport  $\text{Ca}^{2+}$  in synthetic liposomes (Nagaraj *et al.*, 1980; Iqbal, 1982). Their activity is, however, less than that observed for alamethicin. Peptide aggregation in both aqueous and lipid phases may play an important role in channel formation (Mathew and Balaram, 1983a,b, 1984; Mathew *et al.*, 1982). It has been shown that the ease of aqueous phase aggregation is dependent on peptide chain length, longer peptides associating at lower concentrations than shorter fragments (Mathew *et al.*, 1981b, 1982). The differential effects of the peptides on the lipid phase transition may reflect differences in their surface activity or 'detergent like' properties, with alamethicin acting as a more effective perturbant than its shorter fragments. It should be noted that peptide effects on the lipid phase transition have been monitored at significantly higher peptide-lipid ratios, than those employed in cation translocation studies (Nagaraj *et al.*, 1980; Mathew *et al.*, 1982).

Figure 5 compares the effect of increasing alamethicin concentration on the P values observed for DPH incorporated in DPPC, dimyristoylphosphatidyl choline (DMPC)



**Figure 5.** Influence of alamethicin on the fluorescence polarization of DPH incorporated into different liposomes, (Δ), DPPC; (□), DMPC; (○), egg yolk lecithin. Lipid-peptide suspensions (28:1) were prepared as described in the legend to figure 4. Further increases of peptide concentration were accomplished by injection of 1 μl of an alamethicin stock solution (9.8 mM) into the lipid suspensions, followed by sonication. All other conditions are as described for figure 4.

and egg lecithin vesicles. The phase transition temperatures for these systems, in the absence of peptide are 41°C, 23°C and -7 to -15°C, respectively (Ladbrooke and Chapman, 1969). The experiments summarized in figure 5 were carried out at 25°C. Peptide incorporation lowers the *P* value only in the case of DPPC, but is largely without effect on DMPC and egg lecithin, which are already in a fluid state above their respective transition temperatures. Peptide molecules appear to be easily incorporated into fluid bilayers, without further significant perturbation of lipid packing. This observation is of relevance for studies of peptide interaction with natural membrane systems, where lipid phases are generally in a 'fluid' state.

### Peptide effects on mitochondria

The energy derived from substrate oxidation in mitochondria is utilized for phosphorylating ADP in mitochondria. The chemiosmotic hypothesis visualizes energy transduction *via* an electrochemical  $H^+$  and potential gradient across the inner mitochondrial membrane as a means of coupling the oxidation and phosphorylation reactions (Mitchell, 1966). Substances which break down transmembrane  $H^+$  or ion gradients (protonophores or ionophores) can serve to uncouple oxidative phosphorylation (Green, 1977). Channel forming peptides would therefore be expected to act as uncouplers and indeed one of the earliest characterized uncouplers was the peptide antibiotic, gramicidin A (Hotchkiss, 1944). The initial observation that the natural peptides alamethicin and hypelcin uncouple oxidative phosphorylation in rat liver mitochondria (Takaishi *et al.*, 1980) was extended to demonstrate qualitatively similar activities for synthetic alamethicin and its shorter fragments (Mathew *et al.*, 1981a,b, 1982). Figure 6 shows representative experiments on the effect of various peptide

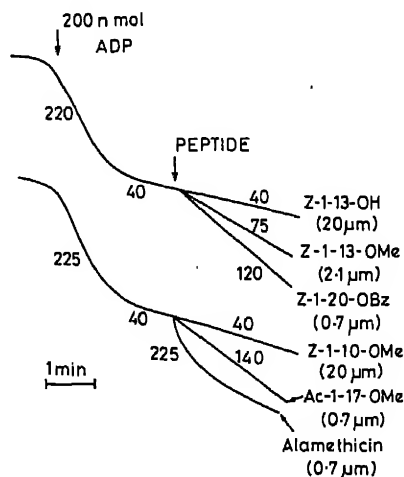
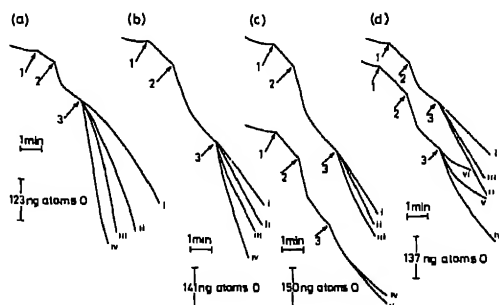


Figure 6. Effect of alamethicin and its fragments on state 4 respiration of rat liver mitochondria. Substrate used was succinate. Numbers shown on traces indicate  $O_2$  consumption in ng atoms  $O/min/mg$  mitochondrial protein. Peptide nomenclature: P-1-*n*-*c* where P is the protecting group, *n* the number of residues in the alamethicin sequence and *c* the C-terminal group. Z = benzyloxycarbonyl, Ac = acetyl, OMe = methyl ester, OBz = benzyl ester (Mathew and Balaram, 1984).

fragments on oxygen consumption by mitochondria. Uncoupling efficiency depends on chain length, with the longer peptides acting at lower concentrations. An interesting feature is that the uncoupling activity of alamethicin is dependent on the concentration of phosphate ions ( $P_i$ ) in the medium. Significant enhancement of respiratory rates is observed at  $P_i$  concentrations between 1 and 10 mM (Takaishi *et al.*, 1980). While  $P_i$  dependent uncoupling has been observed in the case of the cationic cyanine dye uncouplers (Terada, 1979) and peptide carrier ionophores like valinomycin (Moore and Pressman, 1964), the role of  $P_i$  in this process remains to be clearly understood (Terada, 1981).

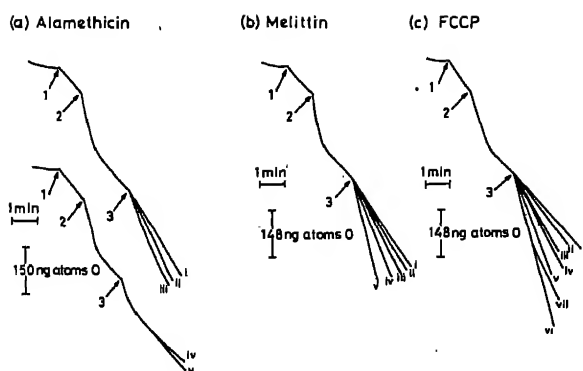
We have therefore examined the influence of a wide range of  $P_i$  concentrations (2.5–100 mM) on the effects of alamethicin on oxidative phosphorylation. Figure 7 shows the effect of alamethicin on state 4 mitochondria at various peptide and  $P_i$  concentrations. Upto 25 mM  $P_i$  alamethicin behaves as an uncoupler, stimulating respiration, at peptide concentrations varying from 0.045–0.3  $\mu$ M. At a higher range of  $P_i$  concentrations (50–100 mM) there is an initial stimulation followed by a distinct reduction in the respiratory rate, suggestive of an inhibitory effect. This tendency is particularly evident in the results for alamethicin concentrations of 0.075–0.15  $\mu$ M, at 100 mM  $P_i$ . A further feature of interest is that at 2.5 mM  $P_i$  and low alamethicin concentration (0.075  $\mu$ M), there is an initial lag period before stimulation of  $O_2$  consumption sets in (figure 7a, trace i).

Figure 8 compares the effect of alamethicin, with those of the bee venom peptide melittin ( $H_2N$ -Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-



**Figure 7.** Effect of alamethicin on state 4 respiration in rat liver mitochondria suspended in media with  $P_i$  concentrations of (a) 2.5 mM, (b) 25 mM, (c) 50 mM and (d) 100 mM. Alamethicin concentrations are: (a) 0.075  $\mu$ M (i), 0.105  $\mu$ M (ii), 0.15  $\mu$ M (iii), 0.30  $\mu$ M (iv). (b) 0.045  $\mu$ M (i), 0.075  $\mu$ M (ii), 0.150  $\mu$ M (iii), 0.30  $\mu$ M (iv). (c) 0.03  $\mu$ M (i), 0.045  $\mu$ M (ii), 0.075  $\mu$ M (iii), 0.15  $\mu$ M (iv), 0.3  $\mu$ M (v). (d) 0.0075  $\mu$ M (i), 0.015  $\mu$ M (ii), 0.03  $\mu$ M (iii), 0.045  $\mu$ M (iv), 0.075  $\mu$ M (v), 0.15  $\mu$ M (vi). Mitochondrial protein concentration  $\sim$  0.48 mg/ml. Mitochondria were isolated from rat liver by the procedure of Pedersen *et al.* (1978) and suspended in 2 ml of assay medium, containing D(–) mannitol (220 mM), sucrose (70 mM), HEPES (2 mM), EGTA (0.5 mM),  $MgCl_2$  (2.5 mM) and  $KH_2PO_4$  at varying concentrations as indicated. pH was adjusted to 7.0. Oxygen consumption was monitored using a Hansatech oxygen electrode at 33°C. Points 1, 2, 3 indicate additions of succinate (7.5 mM), ADP and alamethicin, respectively. ADP was added at concentrations of (a) 72  $\mu$ M, (b) 90  $\mu$ M, (c) 110  $\mu$ M and (d) 77  $\mu$ M. Alamethicin stock solutions were prepared in ethanol and added volumes did not exceed 5  $\mu$ l.

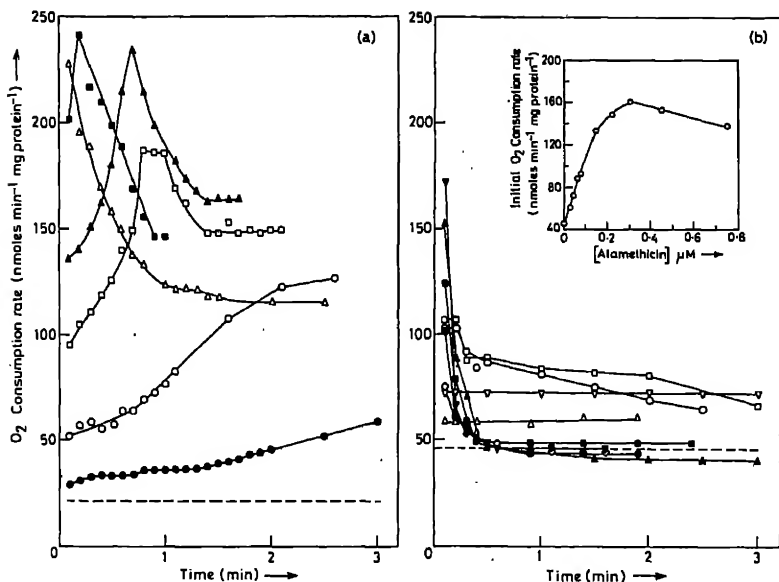




**Figure 8.** A comparison of the effects of alamethicin (a), melittin (b) and FCCP (c) on state 4 respiration of rat liver mitochondria suspended in a medium containing 50 mM  $P_i$ . Alamethicin concentrations (i) 0.03  $\mu$ M, (ii) 0.045  $\mu$ M, (iii) 0.075  $\mu$ M, (iv) 0.15  $\mu$ M, (v) 0.3  $\mu$ M. Melittin (i) 0.30  $\mu$ M (ii) 0.5  $\mu$ M, (iii) 0.75  $\mu$ M (iv) 1.5  $\mu$ M (v) 2.25  $\mu$ M. FCCP (i) 0.003  $\mu$ M, (ii) 0.01  $\mu$ M (iii) 0.0145  $\mu$ M (iv) 0.018  $\mu$ M (v) 0.039  $\mu$ M (vi) 0.051  $\mu$ M (vii) 0.45  $\mu$ M. Mitochondrial protein concentrations (a) 0.49 mg/ml, (b) 0.59 mg/ml and (c) 0.64 mg/ml for i-v and 0.75 mg/ml for vi and vii. ADP concentrations (a) 110  $\mu$ M (b) 110  $\mu$ M and (c) 124  $\mu$ M. All other conditions as described in figure 7.

Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH<sub>2</sub>) and the classical protonophoric uncoupler, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), at a  $P_i$  concentration of 50 mM. Melittin is known to form channels in artificial lipid membranes and is therefore expected to uncouple mitochondria (Tosteson and Tosteson, 1981). Alamethicin at a concentration of 0.15  $\mu$ M shows a distinct reduction in O<sub>2</sub> consumption rate after an initial stimulation. In contrast, both melittin and FCCP do not show any evidence for a reduction in respiratory rate at the concentration studied.

A constant rate of O<sub>2</sub> consumption following peptide addition to state 4 mitochondria results in a linear fall in O<sub>2</sub> level with time, until the anaerobic limit is attained. This kind of behaviour is normally observed for classical uncouplers and is seen in figure 8 for melittin and FCCP. Changes in O<sub>2</sub> consumption rate as a function of time, after uncoupler addition result in non-linear, curved traces. It is useful to analyze this time dependence of O<sub>2</sub> consumption rate. The results of such an analysis are shown in figure 9, for various alamethicin concentrations at  $P_i$  concentrations of 2.5 and 50 mM. Respiration rates have been measured by taking slopes at different time intervals along the O<sub>2</sub> consumption curve. Values have been taken only at points which correspond to total O<sub>2</sub> concentrations of at least 200 nanogram atoms/ml. At these levels there is no inherent dependence of respiration rate on total O<sub>2</sub> concentration. In the case of  $P_i = 2.5$  mM, there is a time dependent increase in O<sub>2</sub> consumption after peptide addition, at low alamethicin concentrations. For 0.18  $\mu$ M peptide there is an initial increase in respiration rate after addition, followed by a decreased rate of O<sub>2</sub> consumption which appears at shorter times after peptide addition and also at higher alamethicin concentrations. Figure 9b demonstrates a marked difference in the observed behaviour at  $P_i = 50$  mM. For alamethicin concentrations below 0.75  $\mu$ M the



**Figure 9.** Time dependence of O<sub>2</sub> consumption rate following alamethicin addition to state 4 mitochondria. (a) Assay medium containing 2.5 mM KH<sub>2</sub>PO<sub>4</sub>. Alamethicin concentrations (●), 0.075 μM; (○), 0.105 μM; (□), 0.18 μM; (▲), 0.225 μM; (■), 0.49 μM; (△), 1.05 μM. Mitochondrial protein 0.47 mg/ml. (—) indicates normal state 4 respiration rate. (b) Assay medium containing 50 mM KH<sub>2</sub>PO<sub>4</sub>. Alamethicin concentrations (△), 0.03 μM; (▼), 0.045 μM; (□), 0.06 μM; (○), 0.075 μM; (▲), 0.15 μM; (▼), 0.225 μM; (●), 0.30 μM; (■), 0.45 μM; (◇), 0.75 μM. Mitochondrial protein 0.49 mg/ml. --- indicates normal state 4 respiration rate. *Inset:* Dependence of O<sub>2</sub> consumption rate immediately after alamethicin addition (initial slope) on peptide concentration.

respiration rate shows little time dependence. However, at higher peptide concentrations there is a sharp initial enhancement of respiration following peptide addition, with a rapid subsequent drop in the rate. At an alamethicin level  $\approx 0.3 \mu\text{M}$  a drop below the normal state 4 respiration rate is observed, suggestive of an inhibitory effect. Figure 9b (inset) shows the influence of alamethicin concentration on the initial O<sub>2</sub> consumption rate, measured immediately after peptide addition. The initial rate increases with alamethicin concentration up to  $0.3 \mu\text{M}$ , while at higher concentrations a decrease sets in.

Phosphate ion concentrations in the medium thus appear to modulate the effects of alamethicin on mitochondrial oxidative phosphorylation. At low  $P_i$  levels the uncoupling effect presumably arises due to formation of transmembrane channels, which can dissipate the proton electrochemical gradient across the inner mitochondrial membrane. At high  $P_i$  concentrations  $> 10 \text{ mM}$ , mitochondria begin to swell (Hunter and Ford, 1955), with a steady increase in the swelling rate and extent of swelling up to  $100 \text{ mM}$  (unpublished). Even though lower RCI values are obtained at  $100 \text{ mM } P_i$  ( $\sim 4.4$ ) as compared to  $2.5 \text{ mM } P_i$  ( $\sim 6.7$ ), a significant degree of coupling is still obtained at high  $P_i$  levels. Since mitochondrial swelling follows changes in membrane

permeabilities, it is reasonable to expect that swelling may also be accompanied by changes in the mobilities and accessibilities of membrane bound components of the electron transport chain. We speculate that the inhibitory action of the peptide, at high  $P_i$  concentrations, may be due to direct binding to protein components of the electron transport system. Helix macrodipole interactions have been involved to rationalise formation of alamethicin aggregates in lipid bilayers (Mathew and Balaram, 1983b). It is conceivable that such helix dipole-dipole interactions may mediate the binding of helical peptides like alamethicin to transmembrane, helical segments of mitochondrial membrane proteins. Since uncouplers like FCCP did not reverse the inhibitory effect of alamethicin, the peptide does not appear to act by inhibiting ATP synthetase activity. It is pertinent to note that the related polypeptides, efrapeptin (Cross and Kohlbrenner, 1978) and elvapeptin (Bullogh *et al.*, 1982) have been shown to inhibit soluble  $F_1$ -ATPase.

A working hypothesis to rationalize the effects of alamethicin on mitochondria is presented below, which may serve to stimulate the design of further experiments in this area. The initial increase in respiration rate on peptide addition (figure 9) could result from incorporation of alamethicin into the lipid phase followed by channel opening. The subsequent drop in  $O_2$  consumption presumably arises due to inhibition of a fraction of respiratory chains, by direct interaction of peptide. The probability of such inhibitory interactions increases with alamethicin concentration, as evidenced by a shift of the peak in the  $O_2$  consumption rate to shorter times after addition of peptide. At high  $P_i$  concentrations ( $\geq 50$  mM) peptide incorporation is very rapid and no peak in respiration rate is observed. The time lag observed for the onset of inhibitory effects is probably determined by the diffusibility of alamethicin molecules in the membrane plane, which is necessary to reach the site of interaction from the site of insertion of the peptide.

Alamethicin and related natural polypeptides provide structurally well defined systems for investigating peptide lipid interactions. Further studies of alamethicin effects on mitochondria may clarify the molecular mechanism underlying the uncoupling and inhibitory effects manifested by this peptide.

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## Proline peptide isomerization and protein folding

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**Abstract.** The unfolding-refolding of proteins is a cooperative process and, as judged by equilibrium properties, occurs in one step involving the native,  $N$ , and the unfolded  $U$ , conformational states. Kinetic studies have shown that the denatured protein exists as a mixture of slow- ( $U_S$ ) and fast- ( $U_F$ ) refolding forms produced by proline peptide *cis-trans* isomerization. Proline residues in  $U_F$  are in the same configuration as in the native protein while they are in non-native configuration in  $U_S$ . For protein folding to occur quickly  $U_S$  must be converted into  $U_F$ . The fact that the equilibrium and kinetic properties of  $U_S \rightleftharpoons U_F$  are the same as those found for proline *cis-trans* isomerization taken together with the absence of slow phase in the kinetics of refolding of a protein devoid of proline, support this view. However, the absence of a linear correlation between half-time of reactivation of denatured enzymes and their proline-contents, as well as the dissimilarities in the kinetic properties of  $U_S \rightleftharpoons U_F$  in unfolding and refolding experiments are not consistent with the model. Conformational energy calculation and experimental results on refolding of proteins suggest that some proline residues are non-essential. They will not block protein folding even in wrong isomeric form. The native-like folded structure with incorrect proline isomers will serve as intermediate state(s) in which these prolines will more readily isomerize to the correct isomeric form. The picture becomes more complex when one considers the consequence of *cis-trans* isomerism of non-proline residues on protein folding.

**Keywords.** Protein folding; *cis-trans* proline isomerization.

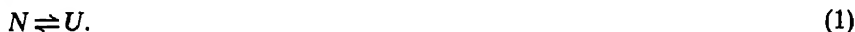
### Introduction

Protein folding is a complex process through which the protein polypeptide chain acquires its native conformation under physiological conditions. What type of native structure a protein molecule will form is specified in its amino acid sequence which in turn is dictated by the specific base sequence of DNA. Why should a protein polypeptide chain fold? An eucaryotic cell cannot accommodate 3-4 million different polypeptides if all of them decide to exist in an unfolded form. Besides, unfolded proteins with exposed peptide bonds will be subject to enormous proteolytic hazards. Further, formation of a crevice in a protein molecule which serves as an active centre recognizing specific substrates/ligands is inconceivable without protein folding. Experimental approach to the problem of protein folding includes studies on unfolding and refolding reaction which involves the interconversion of the native ( $N$ ) and the unfolded ( $U$ ) conformational states of a protein. The native conformation is often compact and globular in which the protein molecule exhibits its characteristic

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Abbreviations used: NMR, nuclear magnetic resonance; BPTI, bovine pancreatic trypsin inhibitor.

biological activity, whereas the unfolded state is devoid of any element of native structure and exists as a structureless random coil (Tanford, 1968). Thus the reversible unfolding of proteins can be expressed as



For a number of relatively low molecular weight globular proteins the transition from  $N$  to  $U$  approximates to a two-state mechanism (Tanford, 1968, 1970; Privalov and Khechinashvili, 1974). The latter implies that states other than  $N$  and  $U$ , if any, exist only transiently and accumulates in quantities too low to have any marked effect on the properties (observables) that are employed in following the process 1. It should, however, be emphasized that conformational transitions within microstates of a macrostate ( $N$  or  $U$ ) are perfectly feasible. Equilibrium (Tanford, 1968) and calorimetric (Privalov and Khechinashvili, 1974) results support the two-state hypothesis of protein unfolding refolding reaction. In contrast, a large body of kinetic data (Baldwin, 1975) on protein denaturations are not consistent with this hypothesis. The time course of unfolding and refolding reactions for a protein would obey the first order kinetic law if the two-state model is valid. On the other hand the kinetics for the reversible denaturation of several globular proteins (Baldwin, 1975) was not simple first-order but more complex. In fact the unfolding and refolding reactions were shown to occur in two kinetic phases, slow and fast phases (Baldwin, 1975). Two factors, among others, may account for the observed complexity. First, the transition  $N \rightleftharpoons U$  may involve a kinetically stable intermediate state which can be detected in kinetic experiments but not in equilibrium studies. Alternatively, one need not involve the existence of a stable intermediate since the non-first order kinetics for unfolding-refolding reaction of a protein can be obtained if there are more than one unfolded states that differ in the rates of refolding.

As noted above, a fully unfolded protein behaves as a random coil free from any residual native structure, so that the difference between the kinetic behaviour of two different unfolded states cannot be attributed to the presence of varying residual native structures. Do they (unfolded states) refold to give back the same native state? The answer for this question is likely to be in the affirmative for most, if not all, proteins. Why do different unfolded states differ in their rates of folding? This difference according to Brandts *et al.* (1975), arises from the *cis-trans* isomerization of proline residues in the unfolded protein. Thus the biphasic kinetics of refolding observed with several proteins are consistent with the scheme,



where  $U_S$  and  $U_F$  are slow- and fast-refolding unfolded states. The two differ in *cis-trans* configuration about the peptide bonds  $\text{NH}_2$ -terminal to proline residues but are indistinguishable with respect to their spectroscopic properties (Brandts *et al.*, 1975). As  $U_S$  and  $U_F$  do not significantly differ in enthalpy the two forms cannot be distinguished by calorimetric method. Therefore, the process 2 will be judged as a two-state process both by direct [calorimetric (Privalov and Khechinashvili, 1974)] and indirect [spectroscopic (Tanford, 1968)] methods. The fast refolding species ( $U_F$ ) have proline residues in the same configuration (*cis* or *trans*) as they do in the native state,  $N$ , whereas the proline residues in the slow-refolding forms ( $U_S$ ) exist in non-native or 'wrong'

configuration. The proline residues in non-native forms in  $U_S$  must isomerize to the correct native configuration before the latter can refold to give the state  $N$  according to 1, 2. Therefore, the conversion of  $U_S$  into  $U_F$  would involve *cis-trans* isomerization, which as we shall see below is a slow process. Kinetic experiments have shown that proteins unfolded by acid and/or denaturant exist as a mixture of fast- and slow-folding forms (Baldwin, 1975, 1978; Schmid and Baldwin, 1978; Kim and Baldwin, 1982). Structural constraints would greatly reduce the possibility of such *cis-trans* isomerism in the native state,  $N$ .

### Proline isomerization

What are the lines of evidence that support *cis-trans* isomerization of an isolated proline in an unfolded protein? As proton or carbon nuclear magnetic resonance gives separate signals (resonances) for the *cis* and *trans* isomer this technique is ideally suited to investigate this issue. Based on nuclear magnetic resonance (NMR) data, it has been shown that proline-containing peptides exist as a mixture of *cis* and *trans* isomers (Brandts *et al.*, 1975). Although the fraction of *cis* isomer appears to depend on nearby ionizable groups, bulkiness of the neighbouring residue and on the nature of solvent, available data suggest that an isolated proline in an unfolded polypeptide chain might possess about 10–30% *cis* character. Thus using  $[^{13}\text{C}]$ -NMR it was found that *cis* isomer in Ala-Pro is 11% for the cationic form of the dipeptide and 35% for the zwitter-ionic form. The ratio, *cis/trans*, is independent of temperature and the two isomers appears to have the same enthalpy.

The kinetics of *cis-trans* isomerization of proline containing dipeptides in aqueous solution was first studied by Brandts *et al.* (1975) using pH jump technique in which the pH of an acidified (pH 1.8) dipeptide solution was raised suddenly (*i.e.* pH jump) by quickly adding a requisite volume of 1 M KOH; the transient pH can be followed by a pH meter equipped with a stripchart recorder. The dipeptides used were Gly-Pro, Ala-Pro, and Val-Pro; in which the bulkiness of the side chain of the *N*-terminal residue increases from Gly-Pro to Val-Pro. For a given peptide, the relaxation time for the *cis-trans* isomerization decreased substantially upon lowering the pH between pH 5 and 3. The relaxation time for zwitter-ionic Ala-Pro at 22.5°C was 300 s while that for the cationic dipeptide was about 75 s. The isomerization process was expectedly slowest with Val-Pro followed by Ala-Pro and Gly-Pro due to the increase in the bulkiness of the adjacent residue. From the dependence of the relaxation time on temperature an activation energy,  $E_a$  of 19.8 Kcal was computed for Ala-Pro (Brandts *et al.*, 1975). Similar  $E_a$  values have been found for polyproline. However, it would not be safe to extrapolate quantitatively the conclusions based on studies on model proline peptides to isolated prolines in an unfolded proteins. But the conclusion that the proline isomerization is a slow process at room temperature would be true for proteins also.

### Interconversion of slow ( $U_S$ ) and fast ( $U_F$ ) refolding forms of proteins and proline *cis* $\leftrightarrow$ *trans* transition

There are several features of the slow phase,  $U_S \leftrightarrow U_F$ , of the protein refolding process which are shared by proline isomerization. For several proteins the activation energies



for the slow phase lie in the range 16–20  $K_{cal}/mol$  while the corresponding values for model proline containing peptides range from 16 to 23  $K_{cal}/mol$ . The relaxation time at 25°C for *cis-trans* proline isomerization is 10–100 s (Schmid and Baldwin, 1978), whereas that for the process  $U_S \rightleftharpoons U_F$  is 8–55 s for six small proteins (Brandts *et al.*, 1975). Other similarities between proline isomerization and  $U_S \rightleftharpoons U_F$  reaction for the best studied protein, ribonuclease A are summarized in table 1. Thus both equilibrium and kinetic properties showed high degree of similarity between the two processes.

Table 1. Properties of proline isomerization and  $U_S \rightleftharpoons U_F$  reaction of ribonuclease A\*.

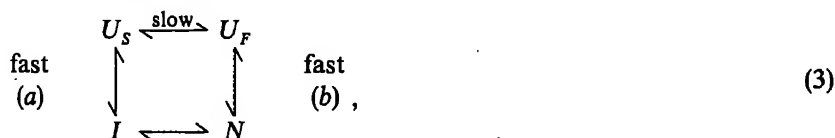
Property	Proline <i>trans</i> $\rightleftharpoons$ <i>cis</i>	$U_S \rightleftharpoons U_F$
Equilibrium constant, $K_{eq}$	0.1–1.0	0.25
Enthalpy change, $\Delta H$	0–1.0 $K_{cal}/mol$	0 $K_{cal}/mol$
Dependence of $K_{eq}$ on pH	Nil	Nil
Dependence of $K_{eq}$ on guanidine hydrochloride concentration	—	Nil
Relaxation time, $\tau$ at 25°	10–100 s	40 s
Dependence of $\tau$ on pH	Nil	Nil
Dependence of $\tau$ on guanidine hydrochloride	Nil	Nil
Activation energy, $K_{cal}/mol$	16–23	21
Acid catalysis	In strong acid	In $HClO_4 \geq 5M$

\* Taken from Schmid and Baldwin (1978).

The experimental evidence for the involvement of *cis-trans* isomerization of proline residues in  $U_S \rightleftharpoons U_F$  process has been provided, among others, by the kinetics of denaturation of parvalbumin (band 5) which is devoid of any proline residues. The characteristic slow-phase with relaxation time of 10–100 s seen with the refolding of other globular protein (see table 1) could not be found in the denaturation of parvalbumin (Brandts *et al.*, 1977; Lin and Brandts, 1978). Although, two fast kinetic phases were detected in parvalbumin denaturation the slowest one was about 100–500 times faster than that found for the denaturation of proline-containing globular protein (see table 1). Further support to the *cis-trans* proline isomerism model comes from the results on the kinetics of slow phase reactivation of denaturated enzymes (Stellwagen, 1979). The half-times for the reactivation process was found between 9 s and 726 s. The activation energy for the reactivation of denaturated adenosine deaminase was determined to be 19  $K_{cal}/mol$  which is similar to that found for the process,  $U_S \rightleftharpoons U_F$  (see table 1).

Some of the basic assumptions of Brandts *et al.* (1975) proline isomerism model that adequately explain the formation of slow refolding species ( $U'_S$ ) according to eq. 2 above are as follows: (i) each proline residue will have an unique *cis* or *trans* configuration in the native protein where *cis-trans* isomerism would be negligible due to structural constraints; (ii) in the denatured protein, isomerization would give a mixture of *cis* and *trans* forms; (iii) each proline residue in the unfolded protein must be in the same configuration as in the native protein before refolding occur. Once the proline peptides in the denatural proteins are in proper isomeric forms protein

molecules would quickly acquire their native conformation. It should, however, be emphasized that the model predicts that the kinetic properties of  $U_S \rightleftharpoons U_F$  would be the same under native and denaturing conditions. But this prediction was found by actual experimentation to be not true (Schmid and Baldwin, 1978; Kim and Baldwin, 1982; Cook *et al.*, 1979). While in unfolding experiments the kinetics features of  $U_S \rightleftharpoons U_F$  were similar to those of proline *cis-trans* isomerization the kinetic properties in the refolding experiments were complex and showed dependence on pH, temperature and denaturant concentration. Further, the model predicts that greater the number of proline residues in a denatured protein/enzyme slower will be the rate of protein unfolding or reactivation. However, this prediction was not supported by actual experiments (Stellwagen, 1979). These results can be reconciled with the Brandts' proposal if the third assumption is modified to read that "each essential proline residue in the unfolded protein must be in the same configuration as in the native protein before refolding occurs". This would mean that certain proline residues in a protein are essential and others are non-essential for the formation of folded protein conformation (Kim and Baldwin, 1982; Baldwin and Creighton, 1980). For protein folding to occur non-essential proline residues in a denatured protein need not isomerize to their native form. Even the 'wrong' isomers can be accommodated in the native structure; they will not block the rapid protein folding. Refined X-ray data along with results on refolding of denatured protein (Baldwin, 1975; Kim and Baldwin, 1982; Baldwin and Creighton, 1980; Levitt, 1981) have demonstrated the existence of such non-essential prolines. Thus proline residues in the terminal sequence, interdomain connectors, and lobes can be tolerated in either configuration (*cis* or *trans*). In view of these considerations, it seems quite possible that segments containing non-essential proline residues in a denatured protein would rapidly fold to give rise to native-like intermediate state *I*, containing non-essential proline in 'wrong' configuration. Results on unfolding of ribonuclease (Cook *et al.*, 1979) taken together with the fact that many large proteins containing proline residues fold much faster (Creighton, 1980) than would be expected from Brandts model are consistent with the view. The eq. 2 for refolding of denatured proteins should, therefore, be revised as follows:



where *I* represents one, or a sequence of intermediate states containing proline peptides in 'wrong' configuration. In the state *I*, the rate of proline isomerization can be enhanced as much as 50-fold. It is closer to the native state in conventional properties that are used in following the denaturation transition but may differ from *N* in other properties (Nall, 1983; Zuniga and Nall, 1983; Muller and Garel, 1984; Schmid and Blaschek, 1984). Both the pathways *i.e.* (a)  $U_S \rightarrow I \rightarrow N$  and (b)  $U_S \rightarrow U_F \rightarrow N$  lead to the formation of the native protein. Which one of the two pathways will prevail? According to Levitt (1981) the refolding route will be determined by the difference in the conformational energies of states *I* and *N*. If the difference is large the pathway (b) will be more important and the refolding process will approximate to Brandts' model. But if the difference is small the first pathway (a) will predominate and significant

amounts of intermediate state (*b*), *I*, will be formed under native conditions.

In bovine pancreatic trypsin inhibitor (BPTI) the four proline residues are in *trans* configuration in the native state, *N*. Proline in *cis* forms can be incorporated in the folded protein with small conformational change to give rise to a native-like state, *I*. Incorporation of a proline residue in 'wrong' isomeric form will destabilize the folded conformation. The strain (or destabilization) energy can be calculated (Levitt, 1981). It turns out, that there are three types of proline residues in BPTI and probably in other proteins. Type I includes those residues which can be freely accommodated in either configuration in the native protein e.g. Pro-13 in BPTI in which case the strain energy for the incorporation of its *cis* form was calculated to be only 1  $K_{cal}/mol$ . Type II proline residues significantly destabilize native protein conformation when incorporated in 'wrong' isomeric forms but the strain or destabilization energy is not so large as to prevent protein folding. Pro-2 and Pro-9 of BPTI for which the strain (or destabilization) energy is 11  $K_{cal}/mol$  belong to this category. Type III proline residues if forced into the native protein as incorrect isomers will destabilize the native protein to such an extent that the protein folding is blocked and can occur only upon isomerization of the proline residues to the correct isomeric forms. For example, Pro-8 in BPTI if inserted in the protein structure in *cis* form will destabilize the protein by 33  $K_{cal}/mol$ . It must isomerize to its *trans* form before refolding can take place. In such situations the refolding of proteins will occur through the pathway (*b*) involving the slow process,  $U_S \rightleftharpoons U_F$  as envisaged in Brandts proposal. The molecules of BPTI having types I and II proline residues will refold much more rapidly than those with type III residues. The proportion of fast refolding BPTI molecules was calculated to be 77% (Levitt, 1981). This is in excellent agreement with the results on refolding on slightly modified BPTI (Jullien and Baldwin, 1981) where the relative amplitude of the fast refolding  $U_F \rightleftharpoons N$  reaction was found to be 75%. Type III behaviour could not be seen with BPTI. It would, therefore, seem that an unfolded polypeptide chain rapidly folds to a compact folded conformation in which proline residues can find their correct native configuration at leisure. However, any generalization in this rapidly changing area is likely to be refuted in future, since we have considered here only the consequences of proline isomerism on protein folding. Isomerization of non-proline peptide bonds, although sterically and energetically less probable (Levitt, 1981; Ramachandran and Mitra, 1976), cannot be ruled out. That such bonds exist in actual proteins is evident from the recent X-ray data on carboxypeptidase A showing three non-proline peptide bonds in *cis* configuration (Rees *et al.*, 1981).

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## Structural mobility and transformations in globular proteins

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**Abstract.** Although globular proteins are endowed with well defined three-dimensional structures, they exhibit substantial mobility within the framework of the given three-dimensional structure. The different types of mobility found in proteins by and large correspond to the different levels of organisational hierarchy in protein architecture. They are of considerable structural and functional significance, and can be broadly classified into (a) thermal and conformational fluctuations, (b) segmental mobility, (c) interdomain mobility and (d) intersubunit mobility. Protein crystallographic studies has provided a wealth of information on all of them. The temperature factors derived from X-ray diffraction studies provide a measure of atomic displacements caused by thermal and conformational fluctuations. The variation of displacement along the polypeptide chain have provided functionally significant information on the flexibility of different regions of the molecule in proteins such as myoglobin, lysozyme and prealbumin. Segmental mobility often involves the movement of a region or a segment of a molecule with respect to the rest, as in the transition between the apo and the holo structures of lactate dehydrogenase. It may also involve rigidification of a disordered region of the molecule as in the activation of the zymogens of serine proteases. Transitions between the apo and the holo structures of alcohol dehydrogenase, and between the free and the sugar bound forms of hexokinase, are good examples of interdomain mobility caused by hinge-bending. The capability of different domains to move semi-independently contributes greatly to the versatility of immunoglobulin molecules. Interdomain mobility in citrate synthase appears to be more complex and its study has led to an alternative description of domain closure. The classical and the most thoroughly studied case of intersubunit mobility is that in haemoglobin. The stereochemical mechanism of the action of this allosteric protein clearly brings out the functional subtleties that could be achieved through intersubunit movements. In addition to ligand binding and activation, environmental changes also often cause structural transformations. The reversible transformation between 2 Zn insulin and 4 Zn insulin is caused by changes in the ionic strength of the medium. Adenylate Kinase provides a good example for functionally significant reversible conformational transitions induced by variation in pH. Available evidences indicate that reversible structural transformations in proteins could also be caused by changes in the aqueous environment, including those in the amount of water surrounding protein molecules.

**Keywords.** Globular proteins; conformational mobility; structural transformations; protein crystallography.

### Introduction

The notion that each globular protein is endowed with a well-defined three-dimensional structure has now been universally accepted primarily as a result of successful X-ray diffraction studies on protein crystals. The crystal structures of well over a hundred globular proteins are currently available. The wealth of information derived therefrom has indeed been crucial in arriving at the current ideas on the structure and action of proteins. However, each X-ray analysis, by its very nature,

essentially gives a time and space averaged static picture of the protein. The spectacular success of protein crystallography has been such that the dynamic aspects of protein structure did not receive as much attention as they deserved, even though early protein crystallographic studies themselves had provided instances of structural changes in protein molecules (Muirhead *et al.*, 1967; Bolton *et al.*, 1968; Quiocho and Lipscomb, 1971). Extensive studies specifically aimed at exploring the structural mobility of proteins are thus of comparatively recent origin. Indeed, spectroscopic, particularly NMR, and theoretical studies have played a major role in developing the dynamic picture of protein molecules (Wuthrich and Wagner, 1979; Wagner, 1983; Ribeiro *et al.*, 1982; Gurd and Rothgeb, 1979; Eftink and Ghiron, 1975; Lakowicz and Weber, 1973; Woodward *et al.*, 1982; McCammon and Karplus, 1983; Levitt, 1983a,b). However, it turns out that a substantial part of the already available definitive information on the dynamical aspects of protein structure has been derived from protein crystallographic investigations. The focus of attention in this review is almost exclusively on the results obtained from such X-ray investigations.

A large number of crystallographic and related studies pertaining to mobility in proteins has been reported in the literature. No effort is made here to survey them exhaustively. The attempt here, perhaps the first of its kind, is to give a connected brief account, using representative examples, of the current understanding, as derived from X-ray analyses, about the mobility in proteins at different structural levels. Also discussed towards the end of the review are X-ray studies on environmental effects on protein structure with particular reference to structural transformations.

Protein molecules are highly complex structural entities involving several levels of organisational hierarchy. Mobility also therefore exists at different levels. The most common and easily understandable motions involve nonspecific rotations about single bonds in amino acid side-chains, especially those in residues occurring on the molecular surface. The other, and more significant, kinds of mobility may be classified as follows:

- (i) Thermal and conformational fluctuations.
- (ii) Segmental mobility.
- (iii) Interdomain mobility.
- (iv) Intersubunit mobility.

The different types of mobility, listed above, are by no means mutually exclusive. Indeed, almost always they coexist. It is, however, instructive to discuss them separately.

### **Thermal and conformational fluctuations**

The X-ray diffraction pattern from a protein crystal, as indeed from any crystal, depends on the atomic positions with respect to a coordinate system defined by the unit cell and the 'temperature factors', usually denoted by  $B$ , of each of the atoms in the structure. The temperature factors would begin to have meaningful values only when the structure has been refined using high resolution, say better than 2 Å, X-ray data. In general, each atom in a well-refined structure is thus associated with coordinates  $x$ ,  $y$ , and  $z$  which define the position of the atom, and a temperature factor  $B$ . The positional coordinates of all the atoms in the molecule define its structure and conformation whereas the  $B$  values contain information on atomic fluctuations.

A crystal of the size suitable for X-ray analysis contains billions of molecules distributed in nearly as many unit cells. If the molecules in all the unit cells have exactly the same structure and conformation and if they are oriented and positioned in exactly the same way, then the image of the molecule averaged over all the unit cells, which is what one obtains from X-ray crystal structure analysis, would be identical to any one molecule in the crystal. In such an ideal situation, each  $B$  value, generally assumed to be isotropic in protein structures, is a direct measure, except for a constant multiplier, of the mean square amplitude of fluctuation or displacement. The time scale of thermal fluctuations is believed to be of the order of picoseconds (Gurd and Rothgeb, 1979) whereas diffraction experiments are performed over a time scale of several hours or days. Hence, what one obtains from X-ray studies are time averaged estimates of fluctuation amplitudes or atomic displacement.

Complications arise when, as is normally the case in protein crystals, all the molecules in the crystal do not have exactly the same structure and conformation. X-ray diffraction provides not only a time averaged, but also a space averaged (over all the molecules in the crystal) image. Thus if a particular atom has a position  $\vec{r}(x, y, z)$  in half the molecules in the crystal and  $\vec{r}'(x', y', z')$  in the other half, peaks at half the normal heights would appear at  $\vec{r}$  and  $\vec{r}'$  in the electron density map when the two atoms are separated by a distance larger than the resolution of the map. If the separation is much smaller than the resolution, the two peaks would overlap giving rise to a single broad peak. Again, the effect of large thermal fluctuations is also to broaden the peak. The effect of static disorder (conformational fluctuations) and dynamic disorder (thermal fluctuations) cannot be separated out in any simple manner, although attempts have been made to do so by carrying out X-ray studies at different temperatures (Frauenfelder *et al.*, 1979; Hartmann *et al.*, 1982). Thus the amplitude of fluctuation or displacement estimated for each atom from high resolution refinement of crystal structures results from the combined effect of conformational and thermal fluctuations. Lattice disorder and translational and rotational diffusion also affect the amplitudes, but as a first approximation, these effects can either be neglected or can be assumed to be constant throughout the molecule (Frauenfelder *et al.*, 1979). Conformational fluctuations and thermal fluctuations are indicative of flexibility, and the displacements of a group of connected atoms give a measure of the flexibility of the region of the molecule made up of these atoms.

High resolution refinement of a number of protein crystals have been carried out in recent years. The structures, the refined parameters of which have been specifically examined for fluctuations, include myoglobin (Frauenfelder *et al.*, 1979), lysozyme (Artymiuk *et al.*, 1979; Sternberg *et al.*, 1979) and prealbumin (Blake and Oatley, 1982). The choice of these three proteins for discussing fluctuations is appropriate as they represent structurally and functionally different families. Myoglobin is a largely  $\alpha$ -helical protein and prealbumin has a  $\beta$ -barrel structure whereas lysozyme is made up of  $\alpha$ -helices as well as  $\beta$ -sheets ( $\alpha + \beta$  structure). Myoglobin is an oxygen storage protein whereas lysozyme is a hydrolytic enzyme. The most well understood function of prealbumin is that of binding thyroid hormones.

Displacements in the three proteins, although they have very different tertiary folds, exhibit striking similarities. As is to be expected, the displacements are in general higher for the surface residues compared to those for internal residues. Also, in general,



hydrophobic residues have lower displacements than hydrophilic residues. These two observations are of course related to a substantial extent. It has been specifically noted in the case of lysozyme and prealbumin that the molecular framework made up of sheets, helices or both have low displacements whereas loops and chain termini are characterised by high displacements. Thus, there is a good correlation, which is eminently sensible from a structural point of view, between structure and displacement in all the three proteins considered. The variation of displacement along the polypeptide chain has also been found to be nearly the same for tetragonal hen egg white lysozyme and orthorhombic human lysozyme despite differences in crystal packing as well as the primary structure; likewise in the structure of prealbumin, variation of displacement along the polypeptide chain is similar in the two chemically identical, but crystallographically independent, subunits in the asymmetric unit. These observations and the correlation between structure and displacement referred to earlier, suggest that the observed displacements represent a molecular property rather than features resulting from crystal packing.

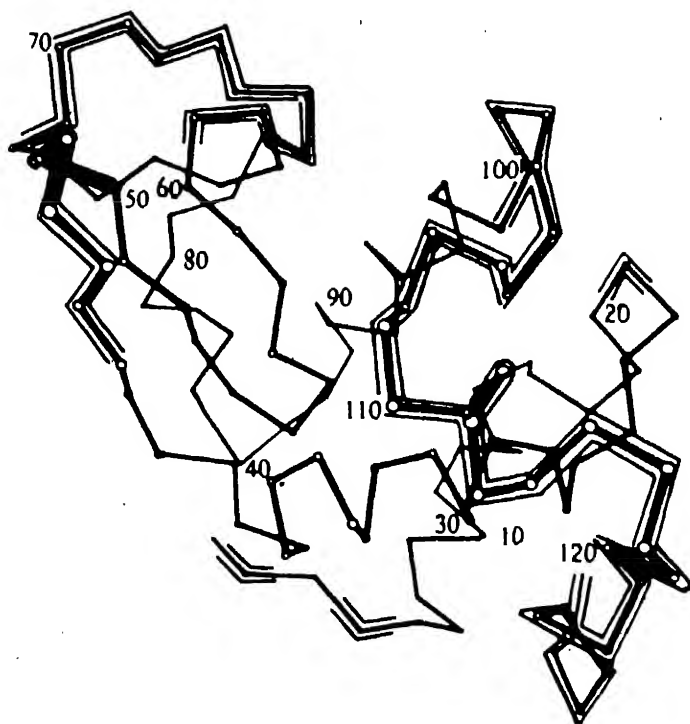
The displacements derived from X-ray analysis appear to have functional significance as well. For example, in myoglobin, fluctuations are low for residues in van der Waals contact with the haem group on the distal side. These residues thus form a comparatively rigid pocket for the oxygen molecule that binds to the haem iron (Frauenfelder *et al.*, 1979). Entrance to the pocket may be possible at the distal side near residues 43–45 which have comparatively high displacement. The same path for the entrance of ligand is suggested by energy calculations as well (Case and Karplus, 1979).

An interesting feature of the displacements in the lysozyme molecule is the observed high fluctuation of the 'lips' of the active site cleft and the regions that are affected by ligand binding (figure 1). In an analogous manner, the outer part of the hormone binding site of prealbumin is also characterised by high displacements (Blake and Oatley, 1982). Presumably, high conformational flexibility of these regions is required for capturing the ligand and guiding it to the binding site. However, flexibility in the neighbourhood of the active site or the binding site does not appear to be a universal feature of protein function. In penicillopepsin, for example, the region around the active site is among those with the least mobility (James *et al.*, 1982).

Several protein dynamics calculations have recently been carried out on structurally well characterized proteins (McCammon and Karplus, 1983). The root mean square displacements obtained from the relevant calculations are in substantial agreement with those obtained from the X-ray work referred to above (Northrup *et al.*, 1980, 1981), thus confirming the reliability of the protein dynamics calculations and the X-ray results. Studies using techniques such as nuclear magnetic resonance (Wuthrich and Wagner, 1979; Wagner, 1983; Gurd and Rothgeb, 1979), which appears to be the method of choice for exploring fluctuations in proteins, fluorescence spectroscopy (Eftink and Ghiron, 1975; Lakowicz and Weber, 1973), solvent exchange (Woodward *et al.*, 1982) and neutron diffraction-hydrogen exchange (Kassiakoff, 1982), also point to the fluctuating nature of protein molecules.

### Segmental mobility

In many proteins, one region or segment of the molecule is more flexible than the others. This mobility often manifests itself in response to ligand binding or activation



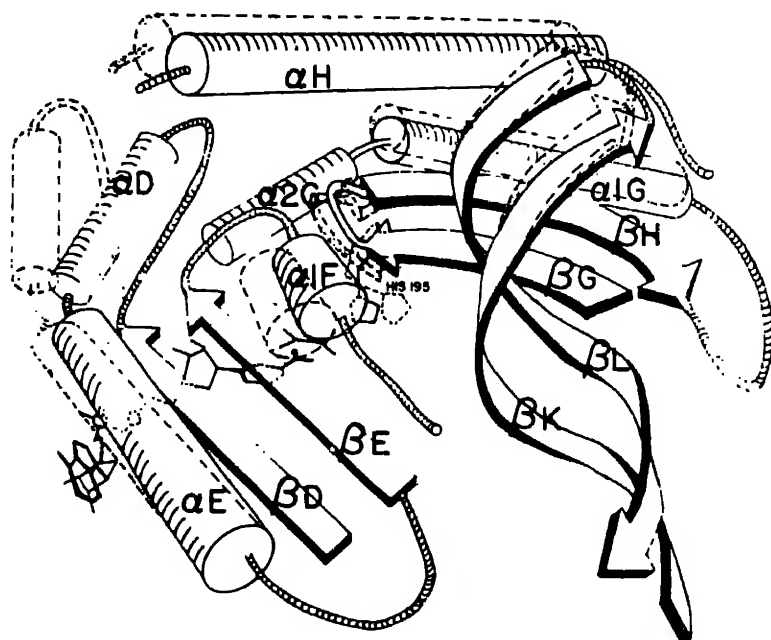
**Figure 1.** A perspective view of the polypeptide chain folding in lysozyme. Regions with residues having high displacement ( $> 0.2 \text{ \AA}^2$ ) in human lysozyme are indicated by the addition of two parallel lines. The high displacement of the two 'lips' of the active site cleft is clearly discernible. Reprinted by permission from *Nature*, 280, 563-568, Copyright © 1979, Macmillan Journals Ltd.

and has considerable functional significance. In many of the early crystallographic studies, small conformational differences, particularly those involving side chains, were observed between a free enzyme and the corresponding enzyme-inhibitor complex. The 'tyrosine-flip' in carboxypeptidase is perhaps a good example of such differences (Quioco and Lipscomb, 1971). Much more dramatic differences, often involving a substantial segment of the polypeptide chain, were subsequently observed in many proteins.

The structure of lactate dehydrogenase provides a good example for pronounced segmental mobility involving considerable difference between the apoenzyme and its complexes (Holbrook *et al.*, 1975). Lactate dehydrogenase is a tetrameric protein involved in the conversion of lactic acid to pyruvic acid with NAD as the coenzyme. Each subunit has two parts, *viz*, the coenzyme binding part and the catalytic part. As the names imply, the former binds the coenzyme whereas the substrate binding site is in the latter.

The crystal structure of the apoenzyme has been determined at 2 Å resolution (Adams *et al.*, 1973). Also analysed are ternary complexes with  $\text{NAD}^+$  and pyruvate, NADH and oxamate, and  $\text{NAD}^+$  and oxalate (White *et al.*, 1976). The molecular architecture is similar in all the structures. Substantial differences in conformation,

however, exist between the apoenzyme on the one hand and the complexes on the other. These differences can be understood by comparing, as in figure 2, the conformations of the apoenzyme and its ternary complex with  $\text{NAD}^+$  and pyruvate. The greatest difference between the two structures pertains to the location, in the coenzyme binding part, of the loop region (residues 98 to 120) which includes the helix  $\alpha\text{D}$ . The loop region, which is on the outside of the molecule, extends into the solvent in the apoenzyme. It moves closer to the main body of the molecule, covers the active centre and encloses the coenzyme and the substrate. Thus a functional active centre, protected from aqueous solvent, is produced by the loop movement.

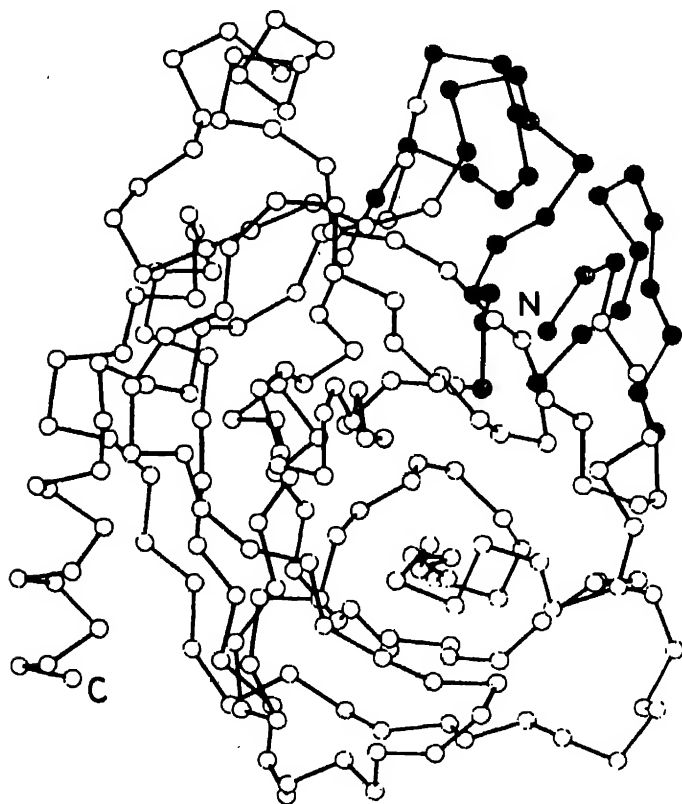


**Figure 2.** A representation of the differences between apo lactate dehydrogenase and its ternary complex. Conformation of the complex is drawn in unbroken lines while that of the apoenzyme is stippled. Reprinted with permission from *J. Mol. Biol.*, 102, 759–779, Copyright © 1976 Academic Press (London) Ltd.

Another striking example of segmental mobility is provided in the conversion, by limited proteolysis, of zymogens of serine proteases to active enzymes. The structural basis of this conversion has been studied in the case of chymotrypsin as well as trypsin. Although the X-ray work on the chymotrypsinogen-chymotrypsin system (Sigler *et al.*, 1968; Freer *et al.*, 1970) was carried out earlier than that on the trypsinogen-trypsin system, we shall consider the latter in some detail as the work on this system has been more extensive and thoroughgoing (Huber and Bode, 1978). As is well known, trypsinogen is converted to trypsin by cleaving the N-terminal activation hexapeptide.

The crystal structures of pancreatic trypsin, trypsinogen and several complexes involving them have been determined at resolutions better than 2 Å (Huber *et al.*, 1974;

Fehlhammer *et al.*, 1977; Bode *et al.*, 1978). The zymogen and the active enzyme have remarkably similar structures except in the region of the so called 'activation domain' made up of the N-terminal residues upto Gly-19, Gly-142 to Pro-152, Gly-184 to Gly-193 and Gly-216 to Asn-223. This domain has an ordered structure in trypsin. No appreciable density exists in the region corresponding to this domain in the electron-density map of trypsinogen indicating thereby that the peptide segments making up the domain have different conformations in different molecules or that they move about in the crystals (Fehlhammer *et al.*, 1977). Thus, the activation domain has a relatively rigid structure in trypsin whereas it is highly flexible in trypsinogen, as illustrated in figure 3.



**Figure 3.** A representation of the structure of the trypsin molecule. Circles represent  $\alpha$ -carbon positions. The  $\alpha$ -carbon positions of residues which are disordered in trypsinogen are shown as filled circles. Atomic coordinates obtained from Cambridge Protein Data Bank were used for preparing this figure.

The catalytic residues Asp-102, His-57 and Ser-195 have nearly the same conformation in trypsin and trypsinogen. The functional difference between the enzyme and the zymogen results from the absence of the specificity pocket in trypsinogen consequent to the flexibility of the activation domain. The rigidification of this domain and the consequent formation of the specificity pocket in trypsin is triggered by a conforma-

tional change in Asp-194 which, on limited proteolysis, forms a salt bridge with the newly formed N-terminus of Ile-16 (Huber and Bode, 1978).

It is interesting to note that trypsin-like conformation, with the rigidification of the activation domain, can be induced in trypsinogen even without the cleavage of the peptide bond adjacent to Ile-16. This can be achieved by complexation with a trypsin inhibitor or by the binding to the zymogen of the N-terminal dipeptide of trypsin, namely, Ile-Val, in the presence of the strong specific inhibitor *p*-guanido-benzoate (Bode *et al.*, 1978).

A transition involving the rigidification of a flexible segment of the molecule, is by no means confined to the activation of the zymogens of serine proteases. A similar phenomenon, induced by RNA binding, occurs in tobacco mosaic virus protein (Bloomer *et al.*, 1978; Stubbs *et al.*, 1977) and pancreatic phospholipase (Dijkstra *et al.*, 1982). It appears that partial disorder in protein molecules could well have direct functional significance also. For example, it has been suggested that, the disorder, as evidenced by the absence of well-defined electron density in the Fourier map, in a part of the tyrosyl tRNA synthetase molecule may correspond to the multiple conformational states involved in different types of catalytic activity (Blow, 1978).

### Interdomain mobility

Many globular proteins are made up of two or more globular autonomous substructures called domains (Wetlaufer, 1973). Lysozyme (Phillips, 1967) and papain (Drenth *et al.*, 1968) were among the first proteins where domain structure was recognised. The active site in both these proteins is situated in the cleft between two domains. Subsequently, organisation of polypeptide chains into domains has been recognised as a basic feature of the molecular architecture of many, particularly large, proteins. For example, all the NAD dependent dehydrogenases have two domains in each of their subunits (Rossmann *et al.*, 1974, 1975). One of them binds the coenzyme and the other the substrate with the active centre located between the domains. The coenzyme binding domain in these dehydrogenases have similar structure whereas the structures of the catalytic domain are different. Immunoglobulin provides another example of the functional significance of domain structure. It contains several domains, each associated with a specific function (Huber, 1983; Davies *et al.*, 1975). There are also situations in which a globular protein contains two or more similar domains as in the case of  $\gamma$ -crystallin (Blundell *et al.*, 1981) and wheat germ agglutinin (Wright, 1977). It has also been possible to establish similarities between domains from different proteins. These similarities have been discussed in terms of the stability of special characteristic chain foldings as well as evolutionary relationships. It has been shown (Richardson, 1981) that domains in proteins identified so far can be broadly classified into a few categories in terms of their structure and topology, suggesting the possibility of considering domains as the basic globular structural elements that go into the design of large proteins or protein subunits. The structure of domains, with particular reference to protein dynamics and function, is a topic of considerable current interest and the work pertaining to it has been reviewed recently (Janin and Wodak, 1983).

Domains in globular proteins, or protein subunits, although connected, are capable

of some restricted independent motion. The extent of possible movement, of course, depends upon the nature of interdomain connections and vary considerably from protein to protein. Interdomain mobility, in addition to being a fascinating structural problem, is also of considerable functional significance as can be seen from the examples discussed below.

Liver alcohol dehydrogenase, an NAD dependent enzyme which oxidises alcohols to aldehydes or ketones, provides a good example for functionally important interdomain mobility (Branden *et al.*, 1975). The enzyme, with two subunits, is a symmetric dimer. Each subunit is made up of two domains with a pocket between them. One of the domains binds the coenzyme while the other binds the substrate with the active site situated in the pocket. The catalytic zinc ion is bound to the catalytic domain well inside the pocket. The dimer formation is mediated exclusively through interactions between the coenzyme-binding domains of the two subunits. In fact the two coenzyme-binding domains together form the core of the dimeric molecule. The two catalytic domains are far apart and flank this core.

The crystal structure of the liver alcohol dehydrogenase apoenzyme and several of its binary and ternary complexes with the coenzyme or analogs and/or substrates or analogs, have been determined (Eklund *et al.*, 1976, 1981). These structures can be broadly classified into two categories in terms of the conformation of the enzyme molecule. The first category is represented by the apoenzyme itself and in general includes complexes involving coenzyme analogs. The second category consists of most of the complexes involving the coenzyme and correspond to the productive mode of coenzyme binding. The ternary complex of the enzyme with NADH and dimethylsulphoxide represents this category. The molecular conformations in the two categories are referred to as the apoenzyme structure and the holoenzyme structure respectively. As can be seen from figure 4, which illustrates the relationship between the two structures, the transition from the apo structure to the holo structure essentially involves a rotation, about the hinge that connects the two domains, of the catalytic domain as a whole by  $7.5^\circ$  (Eklund *et al.*, 1981). The transition is thus a typical case of a 'hinge bending motion'. The core of the dimeric molecule made up of the two coenzyme binding domains remains largely undisturbed except for a small movement in a short stretch in a loop region.

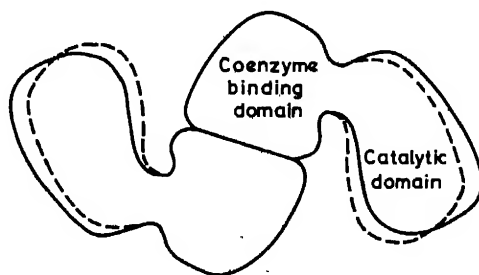


Figure 4. A schematic representation of the apo and the holo structures of the alcohol dehydrogenase dimer. Full lines and broken lines represent apo and holo structures respectively.

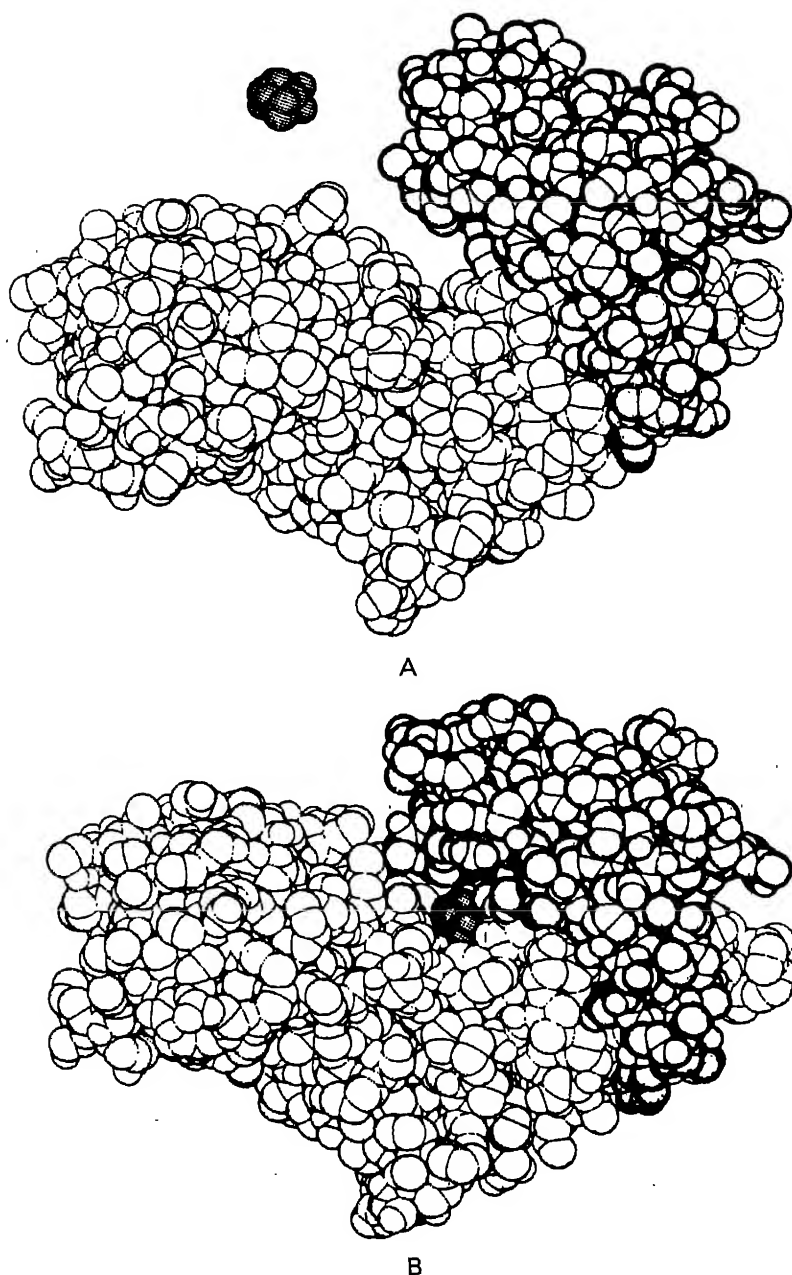
The cleft in the apoenzyme is relatively open. An important consequence of the rotation of the substrate binding domain induced by coenzyme binding is for the cleft to close on the active site region between the two domains. The active site therefore is less accessible from solution and more hydrophobic in the holoenzyme. It is interesting to note that while this effect is achieved by interdomain movement, with the internal structures of the two domains largely preserved, in liver alcohol dehydrogenase, the same effect is achieved in lactate dehydrogenase, discussed in the previous section, through substantial segmental motion involving primarily a loop region in one of the domains.

Yeast hexokinase is another typical protein involving hinge-bending motion. This protein, which catalyses the phosphorylation of glucose by Mg-ATP, exists as two related isozymes, designated *A* and *B*. The structures of several crystal forms of hexokinase and its complexes are available (Steitz *et al.*, 1976; Anderson *et al.*, 1978; Bennett and Steitz, 1980a,b). The enzyme molecule contains two domains, one large and the other small, with the active site in the cleft between them. The glucose binding site is in the middle of the cleft whereas ATP binds to the large domain. The molecule can exist as a dimer or a monomer. In the dimeric molecule, there is an additional ATP binding site between the two subunits.

Substantial conformational differences exist between the apoenzyme and the sugar-bound enzyme. These differences, as deduced from the X-ray analysis of one of the crystal forms of the apo-*B* isozyme and the crystals of the glucose isozyme *A* complex, are illustrated in figure 5. The difference can be described, to a very substantial extent, as a rotation of 12° of one domain with respect to the other about an axis passing through the centre of the molecule. Whereas the cleft is relatively open in the apoenzyme, it closes on the sugar molecule in the glucose-enzyme complex. The molecule in the complex is more compact and the ligand is shielded from solvent (Bennett and Steitz, 1980b). Small angle X-ray studies in solution on isozyme *B* and its sugar complex yield results consistent with the conformational changes on ligand-binding deduced from the single crystal studies as outlined above (McDonald *et al.*, 1979).

The most spectacular example of interdomain mobility is perhaps found in immunoglobulin molecules. As is well known, each molecule is made up of two heavy chains and two light chains. Each heavy chain contains four domains and each light chain two domains. The domains in each chain are linked by flexible, extended polypeptide chains. The flexibility of the interdomain linkages permits the antibody molecule to assume a variety of different conformations. Many of these conformations have been defined through X-ray crystallographic studies (Huber and Bennett 1983; Amzel and Poljak, 1979). The antibody molecule has to deal with a variety of antigens and the flexibility built into its architecture is obviously designed to enable it to do so.

Yet another example of domain mobility is provided by citrate synthase which catalyses the synthesis of citrate from acetyl-coenzyme A and oxaloacetic acid. The structure of this enzyme in an open form and a closed form, in its complex with citrate and coenzyme A, has been described (Remington *et al.*, 1982; Huber and Bennett, 1983). The enzyme has two domains, one large and one small, with a cleft in between them. The cleft is relatively open in the open form. The domains close on citrate and coenzyme A situated in the cleft in the closed form. The difference between the two forms has been explained in terms of a hinge-bending movement. Very recently, the X-



**Figure 5.** Space filling drawings of (A) free hexokinase B and glucose and (B) the hexokinase A: glucose complex. The small domain is more heavily shaded than the large one. Reprinted with permission from *J. Mol. Biol.*, 140 211–230, Copyright © 1980, Academic Press (London) Ltd.



ray analysis of a third crystal form of the enzyme, that of a complex with oxaloacetate formed in the presence of the inhibitor S-acetyl-coenzyme A, has been reported (Wiegand *et al.*, 1984). The molecules in the new form have a closed conformation. The internal structure of the two domains, particularly that of the small domain, in this form, however, deviates significantly from that observed in the open and closed forms described previously, suggesting thereby that the change from open to closed forms could be more complex than a hinge motion (Wiegand *et al.*, 1984).

The open and closed structures of citrate synthase (Remington *et al.*, 1982) have been recently made use of for an alternative description of domain closure (Lesk and Chothia, 1984). According to this description, domain closure, at least in citrate synthase, is caused by the cumulative effect of small shifts and rotations of packed helices. Indeed, this 'helix interface shear' mechanism and the hinge-bending mechanism are not necessarily mutually exclusive. Elements of both could coexist. For example, it has been suggested that domain closure in alcohol dehydrogenase involves features of a hinge mechanism and, in a localised form, the helix interface shear mechanism (Lesk and Chothia, 1984).

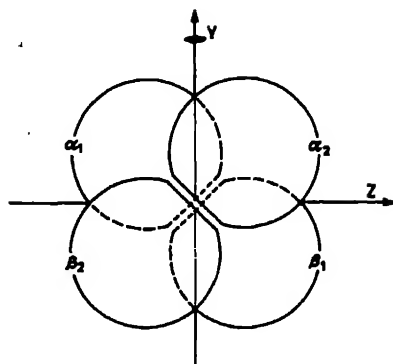
### Intersubunit mobility

Almost all large proteins are oligomeric containing two or more subunits, each made up of an independent polypeptide chain. The arrangement of subunits, the quarternary structure, is generally stabilised by non-covalent interactions. The subunits in a given protein may be identical, similar or totally different. When they are identical or similar, the most frequently encountered exact or approximate symmetry of the assembly is a twofold axis or a combination of three mutually perpendicular twofold axes. Threefold, fourfold or even higher axis of symmetry between subunits is also occasionally encountered. There is at least one instance, in hexokinase, where two subunits are related by a non-integral screw operation (Steitz *et al.*, 1976).

The classic example of intersubunit mobility is that in haemoglobin. The structure and the structural basis of action of no other protein have been studied as thoroughly as those of haemoglobin, thanks mainly to the monumental work of Perutz and his colleagues spanning over close to half a century. Many of their results are now text book material. However, the detailed mechanism of oxygen transport by haemoglobin is still a topic of extensive research. The essential features of the stereochemical basis of this mechanism, though highly complex, now appears to have been established with reasonable certainty.

As is well known, haemoglobin is a tetrameric protein made up of two  $\alpha$  and two  $\beta$  subunits. Each subunit carries a haem group with a central ferrous ion to which an oxygen molecule binds when the protein is oxygenated. The molecule has an exact twofold symmetry which relates the  $\alpha_1\beta_1$  and  $\alpha_2\beta_2$  dimers (figure 6). The two subunits within each dimer are related by an approximate twofold symmetry. Haemoglobin, unlike the closely related myoglobin, is very well known for its cooperative behaviour during ligand binding. This cooperativity is intimately connected to the quarternary structure of the molecule.

It was realised even during the low resolution X-ray analysis that unliganded (deoxy) and fully liganded (oxy) haemoglobin have somewhat different quarternary structures



**Figure 6.** A schematic representation of the haemoglobin tetramer. The molecular twofold axis is along the Yaxis. Reprinted with permission from *J. Mol. Biol.*, **129**, 175–220, Copyright © 1979, Academic Press (London) Ltd.

(Muirhead *et al.*, 1967; Bolton *et al.*, 1968). On the basis of the results of the X-ray analysis of the deoxy and several liganded forms, Perutz proposed his celebrated stereochemical mechanism for the action of haemoglobin in 1970 (Perutz, 1970). This mechanism has been modified and elaborated through several investigations including high resolution X-ray analysis and theoretical studies (Baldwin, 1975; Perutz, 1979; Baldwin and Chothia, 1979; Gelin *et al.*, 1983). This mechanism involves changes, when going from the deoxy form to the liganded form, in the tertiary structure of the subunits and the quaternary structure of the whole molecule. The changes in the tertiary and quaternary structures are indeed coupled.

The liganded or *R* structure and the deoxy or *T* structure have the same internal symmetry. The *T* structure is stabilised, among other non-covalent interactions, by salt bridges involving C-terminal residues of the subunits. These salt bridges, which have been implicated in cooperativity as well as Bohr effect, do not exist in the *R* structure. The interactions that hold together the  $\alpha\beta$  dimer, or in other words the contact region between  $\alpha_1$  and  $\beta_1$  (and the symmetry related  $\alpha_2$  and  $\beta_2$ ), remain largely unaffected during the transition between *R* and *T* states. The main difference between the two quaternary structures can be described as due to a rotation of the  $\alpha_2\beta_2$  subunit as a whole with respect to the  $\alpha_1\beta_1$  subunit. As shown in figure 7, the axis of this rotation intersects and is perpendicular to the molecular twofold axis. It does not however pass through the molecular centre, but is situated in between the two  $\alpha$  subunits at a distance of about 12 Å from the centre.

Each of the two forms (deoxy and liganded) is characterised by its own tertiary and quaternary structures. The tertiary structure of one form is naturally compatible with its quaternary structure. In the deoxy form all the four ferrous ions are five-coordinated. Four coordination sites are occupied by porphyrin nitrogens which lie in a plane. The imidazole ring from the 'proximal' histidine completes the coordination polyhedron which can be described as square pyramidal. No interaction involving the metal ion exists on the other, 'distal' side of the haem group. The ion, in this situation, is displaced from the plane of the porphyrin group towards the proximal side. The oxygen molecule when it binds to haemoglobin does so by coordinating to the haem iron at the

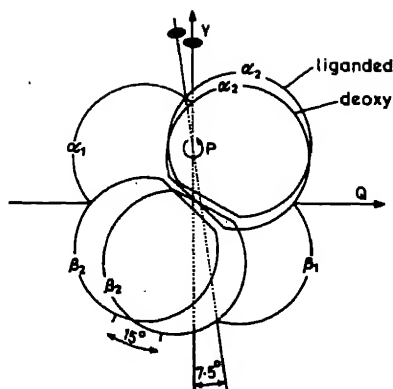


Figure 7. A schematic illustration of the quaternary structural changes in haemoglobin during the transition from the deoxy to the liganded form. P represents the axis about which the  $\alpha_2\beta_2$  dimer rotates with respect to the  $\alpha_1\beta_1$  dimer. Reprinted with permission from *J. Mol. Biol.*, 129, 175–220, Copyright © 1979, Academic Press (London) Ltd.

vacant distal side. The coordination polyhedron is then octahedral, and a strong bond between the metal ion and the ligand is possible only if the ion moves closer to the haem plane. Such a movement would lead to energetically unfavourable steric strain. Hence the lower oxygen affinity of the deoxy structure.

Once a ligand attaches itself to one of the subunits in deoxyhaemoglobin, the steric strain mentioned above has to be relaxed by rearrangement in the tertiary structure in order to form a strong iron-ligand bond. These rearrangements are such as to take the tertiary structure closer to that appropriate for the *R* structure. The atomic movements are transmitted to subunit interfaces causing a partial movement towards the *R* quaternary structure. Now the *T* structure is somewhat relaxed and it is easier for another ligand to bind to a second subunit. This causes a further movement towards *R* structure making it still easier for a third ligand to bind. With the uptake of the fourth ligand, the molecule completely attains the *R* state in which all the four ferrous ions are six coordinated with each ion located in the plane of the corresponding porphyrin nitrogen atoms.

The distances between the four binding sites (ferrous ions) in haemoglobin vary from 23 Å to 39 Å. Ligand binding at one site however affects the affinity of the others. This is achieved not by the direct propagation of atomic movements from one site to another but by a shift in equilibrium between the *R* and *T* states caused by changes in the quaternary structure (Baldwin and Chothia, 1979). Indeed, haemoglobin is the best characterised allosteric protein, although the two state model outlined above differs somewhat from the one proposed by Monod and others (Perutz, 1980; Monod *et al.*, 1965). Also, structural studies on haemoglobin clearly bring out the functional subtleties that could be achieved through intersubunit movements.

Haemoglobin, though the most thoroughly studied, is by no means the only protein in which intersubunit mobility has been characterised by X-ray methods. Another important protein and well known allosteric enzyme in which quaternary structural

changes are currently under investigation is aspartate carbamoyl transferase (Ladner *et al.*, 1982).

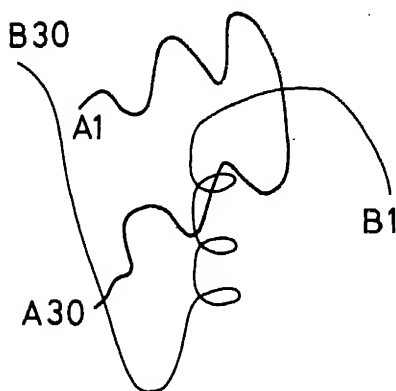
### Structural transformations caused by environmental effects

Structural changes discussed in the last three sections are associated with activation or ligand binding. Striking changes in three-dimensional structure can be caused by changes in the environment such as those in ionic strength and pH as well. Available evidences indicate that changes in the amount and the composition of solvent around protein molecules can also lead to structural transformations.

The protein hormone insulin presents a striking case of a structural transformation due to change in the ionic strength of the medium. The tertiary structure of the hormone was first determined by the X-ray analysis of the crystals of porcine 2 Zn insulin which contains two zinc ions per hexamer (Adams *et al.*, 1969; Blundell *et al.*, 1971). The three dimers in the hexamer are related to one another by an exact three-fold axis while the two monomers in each dimer are related to each other by an approximate twofold axis perpendicular to the exact threefold axis. The two zinc ions are located on the central threefold axis. One molecule from each dimer coordinate through His-B10 to one of the zinc ions while the remaining three molecules, each belonging to a different dimer, coordinate to the other.

The two molecules in the insulin dimer in 2 Zn insulin have nearly the same conformation shown in figure 8. As is well known, pig insulin molecules contain two polypeptide chains, the 21 residue long *A* chain and the 30 residue long *B* chain. The two chains are held together by A7-B7 and A20-B19 disulphide bridges. In addition, there is an internal A6-A11 disulphide bridge in the *A* chain. The *A* chain essentially contains two helices, A1-A9 and A12-A19, which are nearly antiparallel to each other. The *B* chain has a more involved structure. It has an almost extended N-terminal segment followed by the B9-B19  $\alpha$ -helix; the chain then takes a 'U' turn which is followed by an extended C-terminal segment.

A different crystal form of pig insulin, 4 Zn insulin, results when the concentration of sodium chloride in the solution is 6 % or more. The repeat distances and the symmetry



**Figure 8.** A schematic representation of the insulin monomer in 2 Zn insulin. The thick and the thin lines represent the *A* and the *B* chains respectively.

of these crystals are similar to those of 2 Zn insulin. However, their X-ray analysis revealed significant changes in the internal structure (Bentley *et al.*, 1976). 4 Zn insulin crystals, as the name implies, contain 4 zinc ions. One of these is located on the threefold axis while the remaining three are located around it. The two molecules in the dimer are much more dissimilar to each other in 4 Zn insulin than in 2 Zn insulin. One of the two molecules is very similar to those in 2 Zn insulin. The other, however, has a substantially different structure. The major difference between the two molecules pertains to the N-terminal segment of the *B* chain, as can be seen from figure 9. While this segment has a rather extended conformation in 2 Zn insulin, and one of the molecules in the dimer in 4 Zn insulin, it folds and forms an N-terminal extension of the B9-B19  $\alpha$ -helix in the other molecule in 4 Zn insulin. This major conformational change involves atomic shifts up to about 20 Å. It also leads to changes in zinc coordination and in the rest of the molecule. The functional significance of these changes, as indeed the molecular mechanism of insulin action, is still unknown. In any case, it is remarkable that large changes of the type observed in 4 Zn insulin could be brought about, that too reversibly (Bentley *et al.*, 1978), exclusively by changing the ionic strength of the medium.

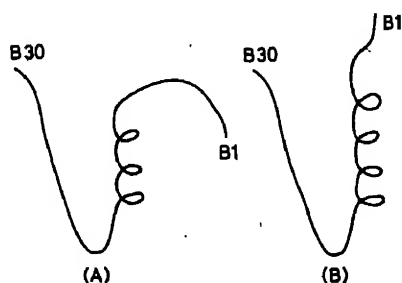
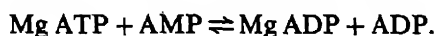


Figure 9. Schematic representations of the *B* chain in (A) the molecules in 2 Zn insulin and one of the monomers in the dimer of 4 Zn insulin and (B) the other monomer in the dimer of 4 Zn insulin.

Adenylate kinase provides a good example for conformational transformations caused by variation of pH. This comparatively small enzyme catalyses the reaction



The enzyme crystallizes in three different, though related, forms depending upon the pH of the medium. Of these three, the X-ray analysis of two forms, designated *A* and *B*, has been carried out (Schulz *et al.*, 1974; Sachsenheimer and Schulz, 1977). *A* form crystallizes in the pH range 6.9–8.0 and *B* form in the range 5.7–5.9, with a hysteresis range of 5.9 to 6.9. A pre-grown crystal of one form can be transformed to the other by appropriately changing the pH of the mother liquor surrounding the crystal.

The overall tertiary fold of the molecule is very similar in *A* and *B*. However, as illustrated in figure 10, substantial conformational difference, involving 15% of the residues, exists between the two forms. The largest change, about 6 Å, occurs in the loop between residues 16 and 22. This change, along with the tilt in the 23–30 helix, opens up a hydrophobic pocket formed by the central  $\beta$ -sheet, the C-terminal helix and 23–30 helix, in the transition from *A* to *B*. Helix 123–133 moves away from the centre of the molecule presumably to accommodate the loop 16–23 in its changed position in *B*. Other, smaller changes, indicated in figure 10, also occur during the transition. On the basis of a close examination of the crystal structures and other lines of evidence, the



affected by substrate binding. In the crystal form *A*, the sulphate ions bound to the enzyme mimic the action of phosphates in the substrates and this explains the existence of the conformation appropriate for  $E^*$  in these crystals even in the absence of substrates.

It appears that structural transformations in protein crystals could be caused by changes in the aqueous environment as well. The earliest experiments bearing on this problem were conducted way back in the late forties and the early fifties when the crystals of haemoglobin were examined under varying conditions of environmental humidity and salt concentration in the mother liquor (Boyes-Watson *et al.*, 1947; Bragg and Perutz, 1952; Huxley and Kendrew, 1953). The results were then explained as due to the movement of molecular layers. The ramifications of the results were not further explored at that time, which is not surprising as protein crystallography was then at its infancy.

Recent experiments in our laboratory on water mediated structural transformations caused by changes in the relative humidity of the environment, suggest that such transformations could well occur in a variety of protein crystals. The crystals on which systematic studies of this type have been carried out include a new crystal form of ribonuclease *A* and the well known tetragonal lysozyme (Salunke *et al.*, 1984). As illustrated in figure 11, the unit cell volume of the new form of ribonuclease *A* reduces discontinuously between 95 and 93% relative humidity when the humidity of the environment of the crystal is reduced systematically. As is well known, all protein crystals contain a substantial amount of solvent which surrounds the protein molecules. In the case of the new form of ribonuclease *A*, 41% of the crystal is made up of aqueous solvent. The discontinuous change in unit cell volume, illustrated in the figure 11, corresponds to a reduction in solvent content to 37% of the crystal volume. This reduction in volume is accompanied by significant changes in the diffraction pattern presumably caused by changes in molecular conformation as well as crystal

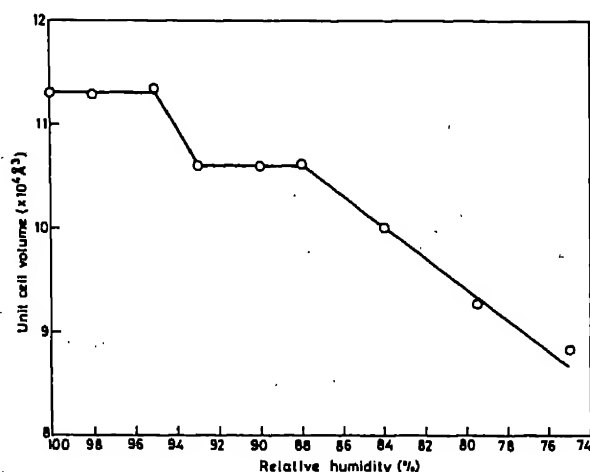


Figure 11. Variation of the unit cell volume in the new crystal form of ribonuclease *A* as a function of the relative humidity of the environment.

packing. Studies on tetragonal lysozyme also yield results similar to those obtained in the case of ribonuclease A. In both the cases, the transformation is reversible in terms of unit cell dimensions and the diffraction pattern. It turns out that this water-mediated transformation is affected by the composition of the solvent that surrounds the crystals. For example, 2-methylpentan 2,4-diol (MPD), when used as a cosolvent, reduces the relative humidity at which the transformation takes place in the new form of ribonuclease A.

Proteins almost always exist and function in aqueous environment. Indeed, hydration of proteins and the effects connected with it have received considerable attention (Careri *et al.*, 1980; Baker *et al.*, 1983; Poole and Finney, 1983; Kuntz and Kauzmann, 1974). In view of the importance of water in the structure and action of proteins, changes in aqueous environment could conceivably have profound effects on protein conformation. Water-mediated transformations provide a useful means for exploring these effects.

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## Bacterial citrate lyase

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**Abstract.** Bacterial citrate lyase, the key enzyme in fermentation of citrate, has interesting structural features. The enzyme is a complex assembled from three non-identical subunits, two having distinct enzymatic activities and one functioning as an acyl-carrier protein. Bacterial citrate lyase, *si*-citrate synthase and ATP-citrate lyase have similar stereospecificities and show cofactor cross-reactions. On account of these common features, the citrate enzymes are promising markers in the study of evolutionary biology. The occurrence, function, regulation and structure of bacterial citrate lyase are reviewed in this article.

**Keywords.** Bacterial citrate lyase; anaerobic citrate utilization; citrate lyase autoinactivation; subunit structure and function; subunit stoichiometry; active sites; citrate lyase prosthetic group.

### Citrate enzymes

Citrate occupies a central position in intermediary metabolism and the enzymes involved in its formation or breakdown have special importance. Four different groups of enzymes are known which catalyze the synthesis (or dissimilation) of citrate through an aldol- (or retroaldol-) type reaction involving an equilibrium between citrate on the one hand and oxaloacetate and an acetyl-moiety on the other. These are:

(i) Bacterial citrate lyase [citrate oxaloacetate-lyase (*pro*-3S-CH<sub>2</sub>COO<sup>-</sup> → acetate); EC 4.1.3.6] found as yet only in procaryotes, which catalyzes the cleavage of citrate into oxaloacetate and acetate in the presence of divalent metal ions such as Mg<sup>2+</sup> or Mn<sup>2+</sup>.



(ii) ATP-citrate lyase [ATP: citrate oxaloacetate-lyase (*pro*-3S-CH<sub>2</sub>COO<sup>-</sup> → acetyl-coenzyme A (CoA): ATP dephosphorylating; EC 4.1.3.8], a cytosolic enzyme responsible for the generation from citrate of 2-carbon moieties which enter a number of biosynthetic pathways. The enzyme, which is present generally in eucaryotes, requires ATP, CoA and a divalent metal ion such as Mg<sup>2+</sup> for the cleavage of citrate.



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Abbreviations used: ACP, Acyl-carrier protein; DTNB, 5,5'-dithiobis(2-nitrobenzoate); DTE, dithioerythritol; *M*, molecular weight; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; CoA, coenzyme A.

(iii) *si*-Citrate synthase [citrate oxaloacetate-lyase (*pro*-3*S*-CH<sub>2</sub>COO<sup>-</sup> → acetyl-CoA); EC 4.1.3.7], which catalyzes the formation of citrate by the overall reaction shown below. It is the most widely distributed of the citrate enzymes and is present in both procaryotes and eucaryotes.



(iv) *re*-Citrate synthase [citrate oxaloacetate-lyase (*pro*-3*R*-CH<sub>2</sub>COO<sup>-</sup> → acetyl-CoA); EC number yet to be assigned] found in some anaerobic bacteria. The enzyme exhibits a stereochemistry opposite to that of *si*-citrate synthase.

Citrate lyase, ATP-citrate lyase and *si*-citrate synthase show close resemblance both in the stereospecificity of the reactions they catalyze and in their cofactor requirements, CoA and the citrate lyase prosthetic group (or their acetyl derivatives) substituting for one another in mediating the three enzyme catalyzed reactions (Robinson *et al.*, 1976). On account of the occurrence of citrate lyase exclusively in primitive forms of life and from its simplest extraneous cofactor requirements, it has been suggested that citrate lyase is perhaps evolutionarily the earliest of these three citrate enzymes (Srere, 1972). Despite this speculation, no sequence data is available even of residues around the active sites of bacterial citrate lyase.

The present review summarizes the literature on bacterial citrate lyase. The earlier work on this enzyme has been reviewed by Srere (1965, 1968, 1972, 1974, 1975), Dagley (1969), Spector (1972) and Srere and Singh (1974).

## Bacterial citrate lyase

### Occurrence

From an analysis of the products of fermentation of citrate by *Klebsiella aerogenes*\* and by *Aerobacter indologenes*, Deffner and Franke (1939) and Brewer and Werkman (1939) concluded that oxaloacetate and acetate are the initial cleavage products. Citrate lyase, the enzyme responsible for this step in the anaerobic utilization of citrate, was characterized first by Dagley and Dawes (1953) in *K. aerogenes* and in *Escherichia coli* and independently by Gunsalus and his group in *E. coli* (Gillespie and Gunsalus, 1953) and in *Streptococcus faecalis* (Grunberg-Manago and Gunsalus, 1953).

Citrate lyase has been found to occur only in bacterial sources, mainly as a citrate-induced activity. The only source in which the enzyme has been found to occur constitutively is *Streptococcus diacetilactis* (Harvey and Collins, 1961). Some strains of *Streptococcus liquefaciens* and *Leuconostoc citrovorum* have also been shown to have citrate lyase activity (Harvey and Collins, 1961). Citrate lyase is present in *Rhodopseudomonas gelatinosa* (Weckesser *et al.*, 1969; Schaab *et al.*, 1972) and *Rhodopseudomonas palustris* (Giffhorn and Kuhn, 1980). *Salmonella typhimurium* grown aerobically on citrate in presence of Na<sup>+</sup> has citrate lyase and oxaloacetate-decarboxylase activities in addition to the enzymes of the citric acid cycle (O'Brien *et al.*, 1969). Citrate lyase has also been reported in *Aerobacter cloacae* (O'Brien and Geisler,

\* Known earlier as *Aerobacter aerogenes*; also known as *Klebsiella pneumoniae* and *Enterobacter aerogenes*.

1974) and in *Proteus rettgeri* (Kröger, 1974) grown anaerobically on citrate. Out of 45 species of *Clostridium* studied: *C. sphenoides* (Walther *et al.*, 1977), *C. sporosphaeroides*, *C. symbiosum*, *C. rectum*, *C. indoles*, *C. subterminale* and *C. sporogenes* (Antranikian *et al.*, 1984) have been shown to utilize citrate as carbon and energy source and to contain citrate lyase. The enzyme from *C. sphenoides* requires the presence of L-glutamate for its activity (Antranikian *et al.*, 1982a), while those from *C. sporosphaeroides* and *C. symbiosum* do not show this requirement (Antranikian *et al.*, 1984). The low enzyme levels in the other species could be detected only indirectly from immunological and [ $1,5\text{-}^{14}\text{C}$ ]citrate experiments.

### Role in metabolism

Citrate lyase functions as the key enzyme in initiating the anaerobic utilization of citrate by a number of bacteria, further catabolism of oxaloacetate formed taking place either by decarboxylation or by reduction. In organisms such as *K. aerogenes*, *S. faecalis* and *R. gelatinosa*, the oxaloacetate is decarboxylated to pyruvate by oxaloacetate decarboxylase, an enzyme which is also induced in these sources in the presence of citrate. The two enzymatic reactions, which occur sequentially, constitute the 'citrate fermentation pathway' (Dagley and Dawes, 1953; O'Brien and Stern, 1969). The subsequent oxidative decarboxylation of pyruvate to acetyl-CoA, a source for the generation of ATP and reducing power, is catalyzed by the pyruvate dehydrogenase complex.

Organisms such as *P. rettgeri* (Kröger, 1974) and *E. coli* (Lutgens and Gottschalk, 1980), however, do not have an oxaloacetate decarboxylase. In these the oxaloacetate formed through citrate cleavage catalyzed by citrate lyase is converted first to malate by the action of malate dehydrogenase and then to fumarate by fumarase. Both *P. rettgeri* and *E. coli* have membrane bound fumarate reductases which produce ATP during the reduction of fumarate to succinate (Hirsch *et al.*, 1963; Bernhard and Gottschalk, 1978). In *P. rettgeri*, the reducing power required for succinate formation is generated by the oxidation of a portion of the citrate through the tricarboxylic acid cycle, in addition to the reducing power one mol ATP being produced per mol of citrate oxidized (Kröger, 1974). In *E. coli*, the tricarboxylic acid cycle is repressed under anaerobic conditions (Amaransingham and Davis, 1965) and the presence of a co-substrate, such as glucose, is required for generation of reducing power. In contrast, *K. aerogenes* does not contain fumarate reductase and depends entirely on oxaloacetate decarboxylase for production of acetyl-CoA (Kulla, 1976).

Evans *et al.* (1966) proposed the reductive carboxylic acid cycle for  $\text{CO}_2$  assimilation by photosynthetic anaerobic bacteria in which citrate lyase was assumed to function as a key enzyme. Beuscher and Gottschalk (1972), however, could not detect citrate lyase activity in *Chlorobium thiosulfatophilum* and *Rhodospirillum rubrum*. ATP-citrate lyase, which had been found earlier only in eucaryotes, has been shown to be involved in the cycle in *Chlorobium limicola* (Ivanovsky *et al.*, 1980; Antranikian *et al.*, 1982b).

### Regulation of citrate lyase

Citrate lyase does not come under regulation in bacteria such as *S. diacetylactis* and *L. citrovorum* in which citrate synthase is not formed (Giffhorn and Gottschalk, 1975a).

However, in bacteria which produce both citrate lyase and citrate synthase, the simultaneous functioning of both enzymes results in energy loss through futile cycles. In such instances, stringent control of the antagonistic enzymes occurs through a variety of regulatory mechanisms. These are briefly reviewed here.

**Regulation in *K. aerogenes*:** O'Brien and Stern (1969) observed that *K. aerogenes* NCTC 418 grows anaerobically on citrate as the sole source of energy only in the presence of  $\text{Na}^+$ . The requirement was explained on the basis of the observation of Stern (1967) that the oxaloacetate decarboxylase of this organism is  $\text{Na}^+$ -dependent. Sachan and Stern (1971a) have shown that the membrane-bound,  $\text{Na}^+$ -requiring oxaloacetate decarboxylase also functions as the carrier protein for the transport of citrate. More recently oxaloacetate decarboxylase from *K. aerogenes* has been shown to function in sodium transport converting the chemical energy of decarboxylation reactions into electrochemical gradients of  $\text{Na}^+$  ions (Dimroth, 1980; 1982a,b). The citric acid cycle is turned off under anaerobic conditions as a result of repression of 2-oxoglutarate dehydrogenase activity (O'Brien, 1975; Wilkerson and Eagon, 1972). Under aerobic conditions of growth on citrate, citrate lyase and oxaloacetate decarboxylase are not induced in *K. aerogenes*, while all the enzymes of the citric acid cycle are formed and cells grown with or without  $\text{Na}^+$  utilise citrate exclusively through this cycle. O'Brien *et al.* (1969) observed that both the pathways are operative when the cells are grown under controlled aerated conditions in which no oxygen tension is detected in the cultures. Under these conditions, citrate degradation proceeds mainly through the fermentation pathway in the presence of  $\text{Na}^+$  and through the citric acid cycle in the absence of  $\text{Na}^+$ .

A mutant of *K. aerogenes* with an altered ionic requirement has been reported recently which unlike the parent strain utilises citrate anaerobically through the citrate fermentation pathway in the presence of  $\text{K}^+$  and in the absence of  $\text{Na}^+$  (Utting, 1983).

A mode of regulation of citrate lyase is through deacetylation of the active acetyl-S-enzyme to a deacetyl (HS)-form, the enzymatic reactivation being catalyzed by a deacetyl citrate lyase ligase in the presence of acetate and ATP. This has been dealt with in detail under 'essential acetyl groups'.

Kulla and Gottschalk (1977) performed a number of growth shift experiments to study the regulation of citrate lyase and citrate synthase activities in *K. aerogenes*. After an initial lag period, citrate lyase activity was found to increase rapidly during anaerobic growth on citrate. No deacetyl citrate lyase could be detected during the early growth phase but formation of the deacetylated enzyme was observed on substrate depletion. Citrate lyase was inactivated by deacetylation on shifting from anaerobic growth on citrate to aerobic growth on glucose or anaerobic growth on glucose in the presence of nitrate. Growth difficulties were noticed on shifting from anaerobic growth on citrate to anaerobic glucose due to the simultaneous functioning of both citrate synthase and citrate lyase. Citrate lyase inactivation was observed to be energy dependent. SivaRaman and SivaRaman (1979) have observed metal cofactor dependent conformational modulations of the enzyme and have suggested that the energy requirement for inactivation could be for the formation or utilization of metabolite(s) which change the conformation of the enzyme either directly or indirectly through complexing of  $\text{Mg}^{2+}$ .

**Regulation in *R. gelatinosa*:** Another mode of regulation of citrate metabolism, besides the induction and repression of enzymes of the two divergent pathways under different growth conditions, is through a loss of the essential acetyl moiety on the citrate lyase through enzymatic deacetylation. While *K. aerogenes* does not contain a citrate lyase deacetylase activity which can inactivate the enzyme (Kulla and Gottschalk, 1977), *R. gelatinosa* has a citrate lyase deacetylase which is regulated by the conditions of growth. *R. gelatinosa* utilises citrate rapidly under anaerobic conditions in the presence of light (Schaab *et al.*, 1972) and the regulation of the two antagonistic pathways has been shown to be through covalent modification and to follow a pattern distinct from that in *K. aerogenes*. The futile cycle is avoided by deacetylation of the enzyme after citrate is completely exhausted from the medium. When citrate is made available, citrate lyase is converted from the inactive deacetylated HS-form to the active acetyl-S-form (Giffhorn and Gottschalk, 1975a, b). The deacetylase has been obtained pure and is a small protein of molecular weight ( $M_r$ ) 14,300 (Giffhorn *et al.*, 1980). The deacetylase of *R. gelatinosa* is highly specific and does not inactivate citrate lyases from *K. aerogenes* and *S. diacetilactis*. The citrate lyase deacetylase is strongly inhibited by L-glutamate (Giffhorn and Gottschalk, 1975b). The intracellular glutamate concentration thus has a regulatory effect on citrate lyase activity. Giffhorn *et al.* (1980) have shown that the intracellular pool size of L-glutamate is in turn proportional to the levels of its precursor, citrate, in the medium. Thus a high concentration of citrate signals that it can be cleaved *via* the fermentation pathway, while at low concentrations, citrate lyase is inactivated by citrate lyase deacetylase and all the citrate is utilised for biosynthesis of L-glutamate. This organism also contains a citrate lyase ligase which activates deacetylated citrate lyase in the presence of ATP and acetate (Giffhorn and Gottschalk, 1975b). The citrate lyase ligase has been purified and shown to have a requirement for ADP for its stability (Antranikian and Gottschalk, 1982). The depletion of citrate from the medium results in inactivation of the ligase and of the citrate lyase activities, while addition of citrate leads to a rapid activation of the ligase which in turn activates citrate lyase (Antranikian *et al.*, 1978).

A similar mechanism of regulation was observed in *R. palustris* (Giffhorn and Kuhn, 1980). Both the enzymes, citrate lyase ligase and citrate lyase deacetylase are present in this organism also and these enzymes were found to cross-react with citrate lyase from *R. gelatinosa*.

**Regulation in *C. sphenoides*:** Antranikian *et al.* (1982a) have reported that the regulation of citrate lyase in *C. sphenoides* is by changes in the intracellular concentration of L-glutamate. The enzyme is active only in the presence of glutamate. While in the *Rhodospirillaceae* the regulatory action of glutamate is indirectly through its effect on the deacetylase, in the *Clostridium* glutamate interacts directly with citrate lyase. The lyase is not deacetylated in the absence of glutamate but apparently is modulated conformationally. Electron microscopy of this citrate lyase did indicate such an effect, the complex being present in undefined structures in the absence of glutamate and as 'star' and 'ring' forms in the presence of the ligand (Antranikian *et al.* 1982a).

**Regulation in *P. rettgeri*:** The oxaloacetate produced by the citrate lyase in this organism is reduced to succinate as oxaloacetate decarboxylase is not formed (Kröger,



1974). It was observed that citrate lyase is induced under anaerobic conditions only with citrate as the growth substrate, while citrate synthase activity is considerably lowered. Besides the induction and repression mechanism, other modes of regulation have not been studied in this organism.

**Regulation in *E. coli* and *S. typhimurium*:** In *E. coli* and *S. typhimurium*, the regulation of citrate lyase activity is through deacetylation. Citrate is utilised in both organisms in the presence of co-substrates and the mode of regulation under anaerobic conditions is by deacetylation of citrate lyase on removal or exhaustion of the co-substrate (Kulla, 1983). It was further shown that citrate lyase could not be deleted from mutants of the LT2 strain of *S. typhimurium* and based on this observation it has been suggested that citrate lyases in *E. coli* and *S. typhimurium* probably function protectively by scavenging citrate which might otherwise chelate divalent cations which are required for cell functions (Kulla, 1983).

#### *Citrate transport*

Citrate uptake in a number of citrate-utilising bacteria has been shown to be mediated by cation-dependent oxaloacetate decarboxylase which is induced in the presence of the substrate. *K. aerogenes* NCTC 418 cells require  $\text{Na}^+$  specifically for anaerobic utilisation of citrate, a requirement which has been attributed to its  $\text{Na}^+$ -dependent oxaloacetate decarboxylase (Stern and Sachan, 1970; Sachan and Stern, 1971a). Mutant strains have been isolated which are  $\text{K}^+$ -dependent, not requiring  $\text{Na}^+$  for citrate uptake (Eagon and Wilkerson, 1972; Utting, 1983). In *S. faecalis*, citrate uptake as well as decarboxylase activity require  $\text{Ca}^{2+}$  and biotin (Sachan and Stern, 1971b). In *S. diacetilactis*, in which citrate lyase is constitutive, the transport system has been shown to be inducible (Harvey and Collins, 1962). Spontaneous cryptic *Cit*<sup>-</sup> mutants of the organism have been isolated which had lost the ability of citrate uptake while retaining citrate lyase activity (Collins and Harvey, 1968). These mutants have been shown either to have lost a plasmid or to have undergone a point mutation despite the presence of the particular plasmid (Kempner and McKay, 1979).

*E. coli* cells do not utilise citrate anaerobically as sole source of carbon and energy. However, the presence of another substrate like glucose, lactose, pyruvate or peptone leads to citrate utilisation under these conditions (Vaughn *et al.*, 1950). The need for a co-substrate such as glucose or lactose has been shown to be for the generation of reducing power from oxaloacetate, since oxaloacetate decarboxylase is not present in this organism (Lutgens and Gottschalk, 1980).

Under aerobic conditions, however, mutants of *E. coli* have been obtained with plasmid-encoded (Sato *et al.*, 1978; Ishiguro *et al.*, 1979; Ishiguro and Sato, 1980; Smith *et al.*, 1978) as well as chromosomal-encoded (Hall, 1982; Reynolds and Silver, 1983) abilities for citrate uptake.

Citrate transport in *S. typhimurium* is found to be inducible and is complex. Three separate transport systems are present in this source for tricarboxylic acids (Imai *et al.*, 1973).

#### *Purification of citrate lyase*

Citrate lyase from *K. aerogenes* has been studied the most since the enzyme was obtained pure for the first time from this source (SivaRaman, 1961).

The general properties of the enzyme purified from diverse sources are summarised in table 1.

Table 1. Properties of purified citrate lyases.

Source	pH optimum	Specific activity ( $\mu\text{mol}/\text{min}^{-1}\text{mg}^{-1}$ )	Molecular properties	
			$s_{20,w}$ (S)	$M_r$
<i>K. aerogenes</i> <sup>a</sup>	8.0	70–90	$s_{20,w}^{20}$ 17.8	575,000 540,000
<i>S. diacetilactis</i> <sup>b</sup>	7.0–7.3	200	16.8	585,000
<i>R. gelatinosa</i> <sup>c</sup>	7.2–7.4	360 (crys)	14.7	530,000– 560,000
<i>S. faecalis</i> <sup>d</sup>	8.0	90	$s_{20,w}^{20}$ 17.1	600,000
<i>C. sphenoides</i> <sup>e</sup>	6.5–7.0	60	16.3	515,000
<i>E. coli</i> <sup>f</sup>	8.0	120	(16.6) <sup>g</sup>	(600,000) <sup>h</sup>
			polydisperse	

<sup>a</sup> SivaRaman (1961); Mahadik and SivaRaman (1968); Bowe and Mortimer (1971); Singh *et al.* (1976); SivaRaman and SivaRaman (1979); Tikare (1979).

<sup>b</sup> Singh and Srere (1975); Kümme *et al.* (1975).

<sup>c</sup> Beuscher *et al.* (1974); Giffhorn and Gottschalk (1978).

<sup>d</sup> Hiremath *et al.* (1976).

<sup>e</sup> Antranikian *et al.* (1982a).

<sup>f</sup> Nilekani and SivaRaman (1983).

<sup>g</sup> Major component

<sup>h</sup> Computed from subunit composition; corresponds approximately to  $M_r$  of major component of  $s_{20,w}$  16.6.

## Stability

Native citrate lyase carries essential acetyl groups and the loss of these by acyl transfer to water is a major mode of enzyme inactivation. That such a loss does occur on storage has been demonstrated by Buckel *et al.* (1971a) who showed that [ $^{14}\text{C}$ ]-acetyl citrate lyase from *K. aerogenes* stored at 4°C for 4 days loses nearly a third of its activity with concomitant loss of an equivalent proportion of protein-bound radioactivity, implicating spontaneous deacetylation as the cause of inactivation. It was also shown that complete inactivation results on deacetylation with neutralized hydroxylamine or DTT/DTE. The inactive enzyme is reactivated chemically with acetic anhydride (Buckel *et al.*, 1971a) or enzymatically with citrate lyase ligase in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974).

The stability of solutions of the pure *K. aerogenes* enzyme has been shown to be greater at higher protein concentrations (Bowen and Rogers, 1963a). The presence of  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  reduces the rate of inactivation of dilute solutions (Eisenthal *et al.*, 1966; Blair *et al.*, 1967), while added bovine serum albumin, sucrose and  $\text{MgSO}_4$  greatly enhance storage stability of lyophilized enzyme preparations (Gruber and Moellering, 1966). The pure *S. diacetilactis* enzyme is stable to storage at  $-90^\circ\text{C}$  (Singh and Srere, 1975). The enzyme from this source was found to be stable at pH values upto 8.1,

dissociation and inactivation of the complex occurring at higher pH values. The purified enzyme from *S. faecalis* was stable when stored at 0°C or -20°C in the presence of ammonium sulphate. Repeated freezing and thawing of the enzyme led to its inactivation with formation of artifacts (Hiremath *et al.*, 1976). The crystalline enzyme from *R. gelatinosa* retains its activity for 8 months when stored at 4°C in the presence of 3 M ammonium sulphate (Giffhorn and Gottschalk, 1978). The purified *E. coli* enzyme is also stable at 0°C in the presence of saturated ammonium sulphate and MgSO<sub>4</sub> (Nilekani, 1980). The pure enzyme from *C. sphenoides* could be stored at 4°C for 4 days or at -20°C for 2 months without appreciable loss in activity, the stability being higher in the presence of L-glutamate (Antranikian *et al.*, 1982a). The loss of enzymatic activity in the absence of L-glutamate could be reversed by its addition. The presence of high concentrations of glycerol (30%), sucrose (40%) or bovine serum albumin (10 mg/ml) together with L-glutamate (7 mM) resulted in only a marginal loss in activity at 0°C. The reversible inactivation in the absence of glutamate was observed to follow a transition from 'ring' and 'star' shapes seen in electron micrographs to undefined shapes.

#### Reaction kinetics

The oxaloacetate formed on cleavage of citrate catalyzed by citrate lyase from *S. diacetylactis* had been characterized as the keto-form by spectral analysis (Harvey and Collins, 1963; Ward and Srere, 1965). The enzyme from *K. aerogenes* has also been shown to yield the keto-isomer in experiments carried out at 2°C and pH 7.4 when spontaneous equilibration of the oxaloacetate tautomers is slowed down (Tate and Datta, 1964). The enzyme thus resembles citrate synthase (Englard, 1959) in the involvement of the keto isomer in the aldol type reaction.

The overall reaction catalyzed by citrate lyase is towards citrate cleavage. The reaction goes virtually to completion in the presence of a large excess of the enzyme (Wheat and Ajl, 1955b; Bowen and Rogers, 1965). The reversibility of the reaction was demonstrated only through radioactive labelling of the citrate using [<sup>14</sup>C]-acetate, oxaloacetate and Mg<sup>2+</sup> in the presence of the enzymes from *S. faecalis* (Gillespie and Gunsalus, 1953), *E. coli* (Wheat and Ajl, 1955b) and *K. aerogenes* (Bowen and Rogers, 1963b).

The equilibrium constant calculated for the cleavage of citrate to oxaloacetate and acetate is about 1 M (Burton, 1955). Several attempts had been made earlier to experimentally determine the equilibrium constant of the enzyme catalyzed cleavage (Smith, *et al.*, 1956; Tate and Datta, 1965; Harvey and Collins, 1963; Guynn *et al.*, 1973). These are of doubtful significance on account of the complexities of the reaction not realized at the time.

#### Product inhibition

Citrate lyases from *E. coli* and *K. aerogenes* have been shown to be irreversibly inhibited by oxaloacetate, a product of the reaction (Dagley and Dawes, 1955). Neither acetate nor pyruvate was found to inhibit the enzyme. The inhibition by oxaloacetate has been shown to be both concentration and time dependent and no changes in spectral or sedimentation profiles could be observed on inactivation (Bowen and

Rogers, 1963b). Malate was also shown to inactivate the enzyme irreversibly while oxalate, succinate, oxaloglutarate, tartarate, oxalosuccinate and isocitrate had no effect.

The inhibition has been shown not to be reversed by malate dehydrogenase and NADH and studies with oxaloacetate and its analogues indicated the requirement of divalent metal ions such as  $Mg^{2+}$  for the inactivation (Eisenthal *et al.*, 1966). In these studies the profile of pH dependence of oxaloacetate inhibition indicated involvement of  $Mg^{2+}$ -enolic oxaloacetate complex which was further supported by  $\alpha,\alpha$ -dimethyl oxaloacetate and ketomalonnate, analogues which cannot enolise, not inhibiting the enzyme. The structural requirement for inhibition were a  $C_4$  straight chain, 1,4-dicarboxylic acid with an ionisable  $\alpha$ -hydroxyl group. Inhibition was also shown to depend on the nature of the divalent metal ion. The inhibitory effect decreased in the order  $Co^{2+}$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ . Inhibition by oxaloacetate has been shown to be through the loss of essential acetyl groups with the formation of the inactive deacetyl citrate lyase (Buckel *et al.*, 1971a).

### Stereochemistry

The stereospecificity of the citrate enzymes has been studied extensively (Eggerer *et al.*, 1970; Retey *et al.*, 1970; Lenz *et al.*, 1971; Wunderwald *et al.*, 1971; Srere, 1975). All citrate enzymes, except *re*-citrate synthase, exhibit similar stereochemistry in regard to the C-3 prochiral centre (Glusker and Srere, 1973) of citrate and the aldol reaction occurs at the *si*-face of oxaloacetate (Hanson and Rose, 1963; Gillespie and Gunsalus, 1953; Wheat and Ajl, 1955b; Buckel *et al.*, 1971a; Srere and Bhaduri, 1964). *re*-Citrate synthase is the only citrate enzyme with an opposite stereospecificity in which the C-1 and C-2 of citrate (*pro*-3*R*- $CH_2COOH$ ) are derived from the acetyl moiety of acetyl-CoA (Gottschalk and Barker, 1966; Stern and Bambers, 1966). All citrate enzymes, including *re*-citrate synthase, however, show identical stereospecificity with respect to the prochiral centres involving the methylene groups on C-2 and C-4 of citrate, causing inversion at the methylene groups which become the methyl groups of acetate (Eggerer *et al.*, 1970; Retey *et al.*, 1970; Klinman and Rose, 1971; Buckel *et al.*, 1971b).

### Metal requirement

Citrate lyases have an absolute requirement for divalent metal ions for catalyzing the cleavage reaction (Dagley and Dawes, 1955; Beuscher *et al.*, 1974; Hiremath *et al.*, 1976; Antranikian *et al.*, 1982a). In the early studies, partially purified enzymes from *K. aerogenes* and *E. coli* were found to be active in the presence of  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Fe^{2+}$ ,  $Co^{2+}$  while  $Ca^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$ ,  $Cu^{2+}$  and  $Hg^{2+}$  did not substitute for the active metal ions (Dagley and Dawes, 1955).  $Ca^{2+}$  was found to act as a competitive inhibitor of enzyme activation by  $Mg^{2+}$ ,  $Mn^{2+}$  and other active metal ions. From these metal ion activation studies, Dagley and Dawes (1955) concluded that metals with a restricted ionic radius of  $0.72 \pm 0.08$  Å can activate the enzyme, while those such as  $Ca^{2+}$  of ionic radius (0.99 Å) outside this specific range have no such effect. The pH optimum of the enzymes was in the range of 7.0–8.2, which approximately parallels the stability of metal-citrate complexes (Bobtelsky and Jordan, 1945). This led to the suggestion that citrate lyase acts on a complex of citrate and divalent metal (Dagley and Dawes, 1955; Harvey and Collins, 1963). Later studies on  $Mn^{2+}$  binding by a partially purified *S.*

*diacetylactis* enzyme preparation using pulsed nuclear magnetic resonance as a probe, however, indicated the formation of a binary complex of metal-protein, no evidence being obtained for a ternary metal-citrate-enzyme complex (Ward and Srere, 1965). It was realized that this could have been due to the steady-state concentrations of such a complex being below detectable levels. The studies, however, confirmed the earlier finding based on kinetic data that  $\text{Ca}^{2+}$  competes with the active metal for its binding site on the enzyme.

Equilibrium dialysis studies have been carried out using radioactive  $^{54}\text{Mn}^{2+}$  as a probe to characterize the nature and the stoichiometry of metal cofactor binding by pure citrate lyase from *K. aerogenes* (SivaRaman and SivaRaman, 1979). The metal binding was found to have no effect on the state of aggregation of the enzyme complex in buffers of adequate concentration. The binding isotherm, Scatchard and Hill plots reflected strong positive cooperativity in metal binding. The estimated number of  $\text{Mn}^{2+}$  binding sites per mol of enzyme complex was 18. More recent studies on the kinetics of inactivation of citrate lyase by 2-fluorocitrates and 2-hydroxycitrates in the presence of different metal ions have also indicated the possibility of metal dependent changes in the conformation of the enzyme (Rokita and Walsh, 1983).

Citrate lyase is a multienzyme complex having two separate enzyme activities, an acyl-transferase activity catalyzing the formation of a citryl-enzyme intermediate and an acyl-lyase activity catalyzing the subsequent cleavage of the citryl intermediate. Only the lyase reaction has been shown to have an absolute requirement for divalent metal ions, while the transferase reaction occurs even in the presence of EDTA (Buckel *et al.*, 1973; Dimroth and Eggerer, 1975b).

### *Essential acetyl groups*

Buckel *et al.* (1971a) were the first to observe that native citrate lyase from *K. aerogenes* is an acetyl-S-enzyme. Stereospecifically labelled  $[5\text{-}^{14}\text{C}]\text{-(3S)citrate}$  was cleaved by citrate lyase to  $[^{14}\text{C}]$ -labelled enzyme and unlabelled oxaloacetate, while  $[1\text{-}^{14}\text{C}]\text{-(3R)citrate}$  yielded unlabelled enzyme and  $[^{14}\text{C}]$ -oxaloacetate. This could be explained as a result of the turnover of acetate during the course of the reaction catalyzed by the enzyme. Thus the overall reaction was recognized to be the sum total of two partial reactions, an acyl exchange reaction:

acetyl-enzyme + citrate  $\rightleftharpoons$  citryl-enzyme + acetate and an acyl-lyase reaction with the cleavage at the *si*-face of oxaloacetate;

citryl-enzyme  $\rightleftharpoons$  acetyl-enzyme + oxaloacetate. Further evidences provided by Buckel *et al.* (1971a) for the involvement of essential acetyl moieties in the reaction mechanism of citrate lyase were: (i) inactivation of  $[^{14}\text{C}]$ -acetyl labelled enzyme by hydroxylamine with formation of  $[^{14}\text{C}]$ -aceto-hydroxamic acid; (ii) the formation of low molecular weight radioactive products and unlabelled protein on treatment of  $[^{14}\text{C}]$ -acetyl-enzyme with cold citrate; (iii) the analogy between hydroxylaminolysis of standard thio-esters like N-succinyl-S-acetylcysteamine and enzyme inactivation by hydroxylamine; (iv) inactivation of citrate lyase by mercaptans like DTT and DTE; and (v) chemical reactivation with acetic anhydride of citrate lyase inactivated by hydroxylamine, mercaptans or oxaloacetate.

Srere *et al.* (1972) showed that citrate lyase inactivated by hydroxylamine or reaction

inactivated in the presence of DTNB could be reactivated only after incubation with DTT. The regeneration of active citrate lyases from the deacetyl form has also been reported for the enzymes from *S. diacetilactis* (Singh and Srere, 1975), *S. faecalis* (Hiremath *et al.*, 1976), *R. gelatinosa* and *L. citrovorum* (Kümmel *et al.*, 1975) and *E. coli* (Nilekani and SivaRaman, 1983), by treatment with acetic anhydride/l-acetyl imidazole.

Treatment of the deacetyl enzyme from *K. aerogenes* with acetic anhydride in the presence of excess of mercaptans results in affinity labelling and consequent reactivation (Dimroth and Eggerer, 1975a). All the sources of citrate lyase examined hitherto have also been shown to have a distinct enzyme, acetate: SH (acetyl carrier protein) citrate lyase ligase (AMP) which catalyzes the reactivation of deacetyl citrate lyase in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974; Hiremath *et al.*, 1976; Bowien and Gottschalk, 1977; Antranikian *et al.*, 1978; Giffhorn and Kuhn, 1980; Antranikian and Gottschalk, 1982; Nilekani and SivaRaman, 1983). The ligases in most instances have been found to be specific for the lyase in the same sources. The *S. diacetilactis* ligase reactivates the lyase from the same source but not the lyases from *K. aerogenes* and *R. gelatinosa* (Bowien and Gottschalk, 1977). The *R. gelatinosa* ligase is active towards the lyases from *Rhodospirillaceae* species but do not cross-react with lyases from *S. diacetilactis*, *K. aerogenes* or *C. sphenoides* (Giffhorn and Kuhn, 1980; Antranikian and Gottschalk, 1982). The ligases from *S. faecalis* and *K. aerogenes*, however, cross-react with the lyases in the two sources (Hiremath *et al.*, 1976) and the *E. coli* lyase cross-reacts with the ligase from *K. aerogenes* (Nilekani and SivaRaman, 1983). The ligase from *K. aerogenes* has also been shown to reactivate deacetyl-citramalate lyase of *Clostridium tetanomorphum*, an enzyme which has close structural resemblance to citrate lyase (Buckel and Bobi, 1976).

### Reaction-inactivation

A characteristic property of citrate lyases is that of autoinactivation during substrate turnover, by acyl transfer to water resulting in the formation of the catalytically inactive deacetyl enzyme. Among the earliest observations were those of Wheat and Ajl (1955b) on the cleavage of citrate by the partially purified *E. coli* enzyme, the reaction stopping rapidly after the initial activity. Inactivation was attributed at that time to inhibition by oxaloacetate. A study of the kinetics of the reaction-inactivation of *K. aerogenes* citrate lyase, however, clearly distinguished between the two modes of inactivation (Singh and Srere, 1971). Preincubation of the enzyme even with a 1000-fold excess of oxaloacetate over the amount formed during the reaction failed to inactivate to a comparable extent. Constant removal of oxaloacetate formed during reaction through coupling with malate dehydrogenase and NADH was also without effect on the inactivation rates. The inactivation rates followed first-order kinetics and was dependent on the nature of the divalent metal ion,  $Zn^{2+}$  causing the slowest rate and  $Mg^{2+}$  the highest among the metals tested. The rapid autoinactivation of the *K. aerogenes* enzyme was termed as a 'suicide' process (Srere and Singh, 1974).

Although the details of the mechanism of reaction-inactivation are not clearly understood, the process is known to be through deacetylation of the native enzyme to the deacetyl-form as the inactivated enzyme is reactivated chemically by acetic

anhydride (Srere *et al.*, 1972) or enzymatically by citrate lyase ligase in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974). Suggested mechanisms for the inactivation are the labile nature of the citryl-S-enzyme intermediate from the observed behaviour of citryl-CoA at alkaline pHs (Buckel *et al.*, 1973); the hydrolysis of the citryl-enzyme intermediate by the acyl-transferase subunit of the enzyme complex (Dimroth and Eggerer, 1975b); and hydrolysis of the mixed anhydride intermediate of acetic and citric acids formed during the transacylation step (Rokita and Walsh, 1983).

Citrate lyases from diverse sources differ in their reaction-inactivation behaviour. The enzymes from sources such as *K. aerogenes* (Singh and Srere, 1971) and *R. gelatinosa* (Kümmel *et al.*, 1975) undergo severe reaction-inactivation, while enzymes from *S. diacetilactis* (Singh and Srere, 1975), *L. citrovorum* (Kümmel *et al.*, 1975) and *S. faecalis* (Hiremath *et al.*, 1976) inactivate at markedly slower rates. The first-order rate constant values for reaction-inactivation of the enzymes from *K. aerogenes*, *S. diacetilactis* (Singh and Srere, 1975), *S. faecalis* (Hiremath, 1977) and *E. coli* (Nilekani and SivaRaman, 1983) with  $Mg^{2+}$  at 25°C were determined to be 1.21, 0.06, 0.07 and 0.32 min<sup>-1</sup> respectively. In the presence of  $Zn^{2+}$ , the values for the *K. aerogenes* and *S. diacetilactis* enzymes were 0.17 and 0.014 min<sup>-1</sup>, respectively.

Citrate lyase autoinactivates also during the turnover of 2-hydroxy- and 2-fluorocitrates (Rokita and Walsh, 1983). While with citrate the frequency of inactivation of *K. aerogenes* citrate lyase was found to be about 1 in 1500 in the presence of 2 mM  $Mg^{2+}$  and 1 in 10,000 in presence of 2 mM  $Zn^{2+}$ , the frequencies were markedly higher with (2R, 3R)- and (2S, 3S)-2-fluorocitrates and the 2-hydroxycitrates, (2S, 3R)-2-hydroxycitrate being the most potent turnover-dependent inactivator deacetylating the enzyme without detectable cleavage of its carbon-skeleton.

### Molecular weight

Citrate lyases have been obtained pure from several sources and shown to be complexes of about  $M_r$  500,000–600,000 (table 1).

The purified *E. coli* enzyme differs from the others in not being monodisperse and exhibiting multiple states of subunit aggregation both in the ultracentrifuge and in polyacrylamide gel electrophoresis under non-denaturing conditions. The sedimentation profile of the *E. coli* enzyme shows multiple peaks with  $s_{20,w}$  values of 10.0, 16.6, 20.4, 23.7 and 30.0 S. The polydisperse behaviour is not effected by a wide range of ionic strengths and pH of Tris and phosphate buffers, presence of divalent metals, mercaptans or EDTA (Nilekani and SivaRaman, 1983).

The enzyme from *K. aerogenes* was shown to dissociate reversibly in buffers of low ionic strength and in the absence of divalent metal ions (Mahadik and SivaRaman, 1968). A half-molecule of  $M_r$  273,000 sedimenting at 10.3 S was observed in 1 mM potassium phosphate buffer, pH 7.4 containing 2 mM EDTA, while another species of 6.3 S was observed in 1 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. Increasing the buffer concentration (50 mM) or including  $Mg^{2+}$  (2 mM) resulted in complete reconstitution of the native species. Treatment with *p*-hydroxymercuribenzoate has also been shown to result in dissociation in stages into three fragments (Mahadik and SivaRaman, 1968). The *S. faecalis* enzyme shows multiple states of aggregation on repeated freezing and thawing (Hiremath *et al.*, 1976) while the *S. diacetilactis* enzyme dissociates at pH values of 8.3–8.7 (Singh and Srere, 1975).

### Subunit structure

The presence of a  $M_r$  10,000 subunit in *K. aerogenes* citrate lyase which functions as an acyl-carrier protein (ACP) was first reported by Dimroth *et al.* (1973). Singh *et al.* (1974) detected by SDS-polyacrylamide gel electrophoresis the presence of three non-identical subunits of 55,000, 33,800 and 11,900  $M_r$  in the enzyme from this source. More precise determination of the molecular weights of the constituent subunits both by gel filtration and sedimentation equilibrium studies in the presence of 6 M guanidinium hydrochloride established that the subunits were of  $M_r$  54,000, 32,000 and 10,000 (table 2). Citrate lyases from *S. diacetilactis*, *S. faecalis*, *R. gelatinosa* and *C. sphenoides* were also shown later to be built up from three different polypeptide chains resembling in size those from the *K. aerogenes* complex. In marked contrast, the *E. coli* enzyme is built up of subunits of 85,000, 54,000 and 32,000  $M_r$  (table 2).

Table 2. Subunit structure of citrate lyases.

Source	Subunits ( $M_r$ )			Holoenzyme structure
	Transferase ( $\alpha$ )	Lyase ( $\beta$ )	ACP ( $\gamma$ )	
<i>K. aerogenes</i> <sup>a</sup>	54,000	32,000	10,000	$\alpha_6\beta_6\gamma_6$
<i>S. diacetilactis</i> <sup>b</sup>	54,000	35,000	12,000	$\alpha_6\beta_6\gamma_6$
<i>S. faecalis</i> <sup>c</sup>	54,000	37,000	14,000	$\alpha_6\beta_6\gamma_6$
<i>R. gelatinosa</i> <sup>d</sup>	55,600	31,600	11,400	$\alpha_6\beta_6\gamma_6$
<i>C. sphenoides</i> <sup>e</sup>	56,000	32,000	11,700	$\alpha_6\beta_6\gamma_6$
<i>E. coli</i> <sup>f</sup>	54,000	32,000	85,000	$\alpha_6\beta_6\gamma$

<sup>a</sup> Carpenter *et al.* (1975); Dimroth and Eggerer (1975a); Singh *et al.* (1976).

<sup>b</sup> Singh and Srere (1975).

<sup>c</sup> Hiremath *et al.* (1976); Hiremath (1977).

<sup>d</sup> Giffhorn and Gottschalk (1978).

<sup>e</sup> Antranikian *et al.* (1982a).

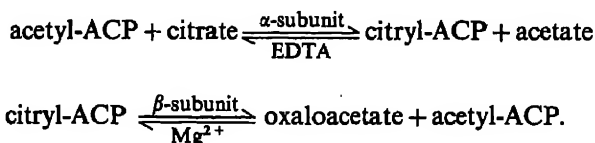
<sup>f</sup> Nilekani and SivaRaman (1983).

### Subunit function

The 10,000  $M_r$  subunits in *K. aerogenes* citrate lyase were shown to contain the covalently-bound 4'-phosphopantetheine moieties which had been detected by Srere *et al.* (1972) in purified holoenzyme from this source (Dimroth *et al.*, 1973). Further evidence for the ACP function of the subunit was provided by dissociation of [ $^{14}\text{C}$ ]-acetyl-citrate lyase and detection of the radioactive label exclusively in the 10,000  $M_r$  component after SDS-polyacrylamide gel electrophoresis (Dimroth *et al.*, 1973). The subsequent detection of three non-identical subunits in the enzyme complex (Singh *et al.*, 1974) and the sequence of acyl-exchange and acyl-lyase steps in the acetyl-CoA mediated cleavage of citrate by the inactive deacetylated complex (Buckel *et al.*, 1973) were suggestive of the presence of two enzymatically active subunits in addition to the ACP subunit. Unequivocal evidence for the multienzyme nature of the *K. aerogenes* citrate lyase complex was provided by the isolation of the three polypeptide chains in



pure and bilogically active states, characterization of their individual functions and reconstitution of the active enzyme from the isolated subunits (Dimroth and Eggerer, 1975b). The 54,000  $M_r$  ( $\alpha$ ) subunit was shown to function as an acyl-transferase catalyzing the formation of the acyl-intermediate, citryl-ACP and acetate from citrate and the 10,000  $M_r$  ( $\gamma$ ) subunit which was earlier shown by Dimroth *et al.* (1973) to function as the ACP. The transferase reaction has no metal requirement and a dimer of the 54,000 ( $\alpha$ ) chain was reported to be the catalytically active form of the subunit. The 32,000  $M_r$  ( $\beta$ ) subunit in the presence of  $Mg^{2+}$  catalyzes the breakdown of citryl-ACP to oxaloacetate and acetyl-ACP, establishing the lyase function of the  $\beta$ -subunit. It was thus established that the overall breakdown of citrate by citrate lyase proceeds in the sequence:



The active enzyme could be reconstituted only in the presence of all three subunits and was identical with the native enzyme in its specific activity and sedimentation behaviour.

Citrate lyases purified from *S. diacetilactis*, *R. gelatinosa*, *S. faecalis* and *C. sphenoides* have been found to resemble the *K. aerogenes* complex in containing subunits of about 55,000, 30,000 and 10,000  $M_r$  (table 2). The smallest subunit has been shown to contain the CoA-like prosthetic group. The largest subunit of about 55,000 $M_r$  has been assumed to function as the acyl-transferase and the smaller subunit of about 32,000  $M_r$  as the acyl-lyase in analogy with the *K. aerogenes* complex. In marked contrast, the citrate lyase complex from *E. coli* has been shown to diverge from the general subunit structure, containing subunits of 85,000, 54,000 and 32,000  $M_r$  (Nilekani and SivaRaman, 1983). The 85,000  $M_r$  subunit has been characterized as the ACP ( $\gamma$ ) subunit both from the covalently-bound prosthetic group components present exclusively on the subunit as well as through reconstitution from the separated subunits. The 54,000  $M_r$  subunit has been characterized as the acyl-transferase ( $\alpha$ ) subunit and the 32,000  $M_r$  as the acyl-lyase ( $\beta$ ) subunit both in acyl-CoA mediated and in acyl-ACP mediated reactions.

Citramalate lyase (EC 4.1.3.22) from *C. tetanomorphum* has been shown to resemble closely *K. aerogenes* citrate lyase despite their occurrence in unrelated bacteria (Buckel and Bobi, 1976). The relatedness between the enzyme complexes is further supported by hybridization experiments. The ACP of citramalate lyase forms an active citrate lyase hybrid complex with the acyl-transferase and acyl-lyase subunits of citrate lyase and the ACP of citrate lyase acts as a substrate for the transferase subunit of citramalate lyase, although other combinations are inactive (Dimroth *et al.*, 1977a).

#### Subunit stoichiometry

The estimates of subunit stoichiometry by scanning of Coomassie blue stained SDS-polyacrylamide gel electrophoretograms indicated equimolar amounts of the  $M_r$  54,000 ( $\alpha$ ) and the  $M_r$  32,000 ( $\beta$ ) subunits in the citrate lyase complex from *K. aerogenes*

(Dimroth and Eggerer, 1975a; Carpenter *et al.*, 1975). The amount of the small ACP ( $\gamma$ ) subunit estimated by this procedure failed to give consistent results, values varying with the development and staining procedures. This was presumed to be caused by losses due to diffusion from the gel of the small polypeptide chain. Singh *et al.* (1976) established a novel sequencing technique in which each of the subunits was sequenced to 10 residues from the amino terminus followed by a similar analysis of amino acids released sequentially at corresponding positions of the holoenzyme. The molar ratios of the amino acids released from the three subunits were shown to be 1 : 1 : 1, indicating the presence of equimolar amounts of the  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits in the enzyme complex. The findings established that the enzyme from the source has a hexameric ( $\alpha_6\beta_6\gamma_6$ ) structure. Estimates of subunits and holoenzyme molecular weights and subunit stoichiometry in the case of citrate lyase complexes from *S. diacetilactis*, *S. faecalis*, *R. gelatinosa* and *C. sphenoides* indicate that these enzymes resemble the *K. aerogenes* enzyme in their gross structural features (table 2). In contrast to the general subunit stoichiometry, the *E. coli* holoenzyme has been shown to have a ratio of  $\alpha$  :  $\beta$  :  $\gamma$  subunits of 6 : 6 : 1.

### Structural organisation

'Ring' shaped structures have been observed in electron micrographs of negatively stained *R. gelatinosa* citrate lyase (Beuscher *et al.*, 1974). These were composed of 6 large and 6 small subunits arranged hexagonally and lying face to face, each ring consisting of 3 large and 3 small subunits in alternating sequence. Similar structural features have been observed with *K. aerogenes* citrate lyase (Dimroth and Eggerer, 1975a; Schramm, 1981). Antranikian *et al.* (1982a) recognized 'star' and 'triangle' forms in addition to the 'ring' forms in electron micrographs of citrate lyases from *C. sphenoides*, *R. gelatinosa* and *S. diacetilactis*. The presence of citrate or its analogue, tricarballoylate, altered the ratio in favour of the 'star' form in case of the enzyme from *S. diacetilactis*. The *C. sphenoides* enzyme, which has an absolute L-glutamate requirement for its activity, showed undefined shapes in the absence of glutamate while the presence of the activator caused the appearance of 'star' and 'ring' forms. The smallest subunit could not be observed in the electron micrographs of the native complexes, apparently because of its small size and the limited resolution of the technique. However, the ACP ( $\gamma$ ) subunit could be located using immunoelectron microscopy and antibodies specific towards the  $\gamma$ -subunit. Under these conditions, the  $\gamma$ -subunit was located to be close to the two larger subunits both in 'stars' and 'rings' with a symmetrical location in the 'star' form and a polar attachment in the 'ring' form (Zimmermann *et al.*, 1982).

### Mechanism of action

**Acyl-transferase activity:** The initial acyl-transferase reaction exhibited by bacterial citrate lyase has been shown to differ from that catalyzed by the well-known CoA transferases represented by succinyl CoA: 3-keto acid transferase in that a single displacement mechanism operates unlike the double displacement of the classical CoA transferases (Jencks, 1973). Kinetic evidences indicated that in citrate lyase an

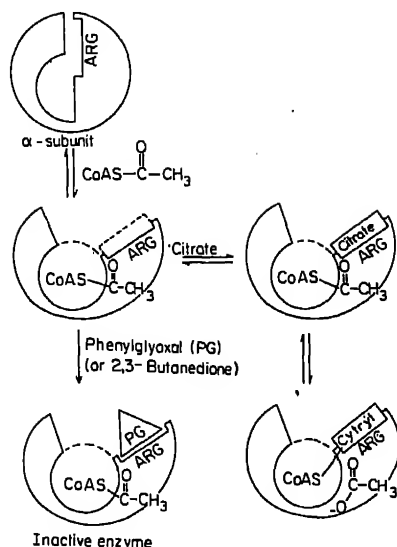
anhydride intermediate between the substrate and the acyl moiety of the prosthetic group is generated without the formation of an enzyme-CoA intermediate in the acetyl-CoA mediated reaction or an acyl-transferase-carbonyl-S-ACP intermediate in the/acetyl-S-ACP mediated step (Dimroth *et al.*, 1977b).

With substrate analogues such as 3-fluoro-3-deoxycitrate, two different modes of reaction-mechanism based inactivation have been observed by Rokita *et al.* (1982). The enzyme was shown to be converted rapidly into equal proportions of two distinct inactive types, a deacetylated form and a 3-fluoro-3-deoxycitryl-S-enzyme. The behaviour of the citrate analogue as a 'suicide' substrate was explained on the basis of the initial formation of an anhydride of the analogue and acetic acid, which either hydrolyzes and leaves the enzyme in the deacetylated form or reacts with the enzyme to yield fluorocitryl-S-ACP, a derivative which structurally cannot undergo retroaldol cleavage but hydrolyzes slowly with the formation of the deacetyl enzyme.

The involvement of a single arginine residue at the active site of the acyl-transferase subunit of citrate lyase has been shown in studies using the arginine specific reagents, phenylglyoxal and 2,3-butanedione as inactivators, both with the complex as well as with the isolated subunit (Subramanian *et al.*, 1983). Citrate and (3*S*)-citryl-CoA protected the transferase activity while acetyl-CoA markedly enhanced the rate of inactivation, reflecting apparently a conformational change at the citrate binding site of the acyltransferase subunit in the presence of acetyl-CoA which makes the essential arginine residue more accessible to  $\alpha$ -carbonyl reagents. An enhancement of reactivity has also been observed in the closely related classical CoA-transferases represented by succinyl-CoA: 3-ketoacid CoA-transferase, the presence of acyl-CoA substrates in this case enhancing the reactivity of an essential thiol group at the carboxylate binding site (White *et al.*, 1976). An 'alligator' model has been suggested for the CoA-transferases (White *et al.*, 1976). The effect of acetyl-CoA on the reactivity of the acyl-transferase  $\alpha$ -subunit of citrate lyase is shown schematically in figure 1 on the basis of such a model.

The photoaffinity reagent *p*-azidobenzoyl-CoA, which specifically inactivates the acyl-lyase subunit of citrate lyase, has been shown to have no effect on the acyl-transferase activity both of the complex as well as of the isolated subunit (Basu *et al.*, 1982).

**Acyl-lyase activity:** The stereochemistry of citrate cleavage has been discussed earlier. The cleavage of the carbon skeleton of hydroxy- and fluoro-citrate isomers which are substrates for citrate lyase has been shown to follow the same regiospecificity, cleavage always occurring at the arm corresponding to the *pro-S*- arm of citrate. In detailed studies on these isomers, Sullivan *et al.* (1977) and Rokita and Walsh (1983) have shown that all isomers of 2-hydroxycitrate, except (2*S*, 3*R*)-2-hydroxycitrate serve as substrates for citrate lyase, while all 4 are linear competitive inhibitors for citrate in the cleavage reaction. Carbon skeletons of (2*R*, 3*R*)- and (2*S*, 3*S*)-2-fluorocitrates were also found to be cleaved catalytically by citrate lyase (Rokita and Walsh, 1983). The stereospecificity of cleavage in all cases was identical to that of citrate, (2*R*, 3*S*)- and (2*S*, 3*S*)-2-hydroxycitrates and (2*R*, 3*R*)-2-fluorocitrate giving oxaloacetate and the glycolyl- or fluoroacetyl form of the resting enzyme, while (2*R*, 3*R*)-2-hydroxycitrate and (2*S*, 3*S*)-2-fluorocitrate yielded  $\beta$ -hydroxyoxaloacetate and  $\beta$ -fluorooxaloacetate respectively, and the acetyl form of citrate lyase. The results were found to support the suggestion of



**Figure 1.** Schematic representation of modulation of activity of the acyl transferase ( $\alpha$ ) subunit of *Klebsiella aerogenes* citrate lyase by acetyl-CoA. The model is based on the alligator-type proposed by White *et al.* (1976) for classical CoA transferases.

Sullivan *et al.* (1977) that only the divalent metal chelate of the 2-hydroxycitrate which is similar to that of citrate leads to cleavage of the carbon skeleton, despite the many possible tridentate 2-hydroxycitrate chelation modes (Stalling *et al.*, 1979).

*p*-Azidobenzoyl-CoA has been shown to act as a potent competitive inhibitor for (3*S*)-citryl-CoA in the citryl-CoA: oxaloacetate lyase reaction catalyzed by deacetyl citrate lyase complex (Basu *et al.*, 1982). The inactivation was irreversible on photolysis and  $\text{Mg}^{2+}$  which is required for the cleavage reaction had no effect on the binding of the inhibitor to the lyase subunit. It was suggested that (3*S*)-citryl-CoA binding is independent of divalent metals, while its cleavage requires the presence of  $\text{Mg}^{2+}$ . The stoichiometry of CoA-ester binding was investigated by the use of *p*-azido [ $^{14}\text{C}$ ]-benzoyl-CoA. During photolysis a linear relationship was observed between incorporation of the [ $^{14}\text{C}$ ]-label and the activity loss under limiting conditions of the reagent, the label being exclusively on the lyase subunit. Under such conditions, the substrate, (3*S*)-citryl-CoA, completely protected the enzyme and prevented incorporation of radioactivity on photolysis. An estimate of active sites per mol of the *K. aerogenes* complex made through this approach indicated that all 6 acyl-lyase subunits are accessible for the acyl-CoA mediated reaction. This would suggest that *in vivo* the cleavage of citrate can proceed both *via* the built-in prosthetic group and through the acyl-CoA mediated reaction.

The acyl-lyase subunit of *K. aerogenes* has also been shown from inactivation kinetics with phenylglyoxal and 2,3-butanedione to contain a single essential arginine residue at its active site (Subramaninan *et al.*, 1983). (3*S*)-Citryl-CoA was found to protect the lyase subunit in the complex from inactivation by the  $\alpha$ -carbonyl reagents, while citrate and acetyl-CoA had no significant effect.

**ACP activity:** The ACP of citrate lyase from *K. aerogenes* (Dimroth *et al.*, 1973) resembles the ACP of fatty acid synthetase from *E. coli* (Vagelos, 1973) in its molecular weight and function. The two, however, have been shown to differ markedly in the nature of their prosthetic groups and in their amino acid compositions. The prosthetic group of fatty acid synthetase ACP is 4'-phosphopantetheine, whereas the ACP of the citrate lyase contains in addition adenine, phosphate and sugar residues (Dimroth, 1976; Robinson *et al.*, 1976). Dimroth *et al.* (1973) have shown that an ACP present in the crude extracts of *K. aerogenes*, but not the purified ACP subunit of its citrate lyase, could substitute for the ACP of the *E. coli* fatty acid synthetase system in the malonyl-CoA/CO<sub>2</sub> exchange reaction. This indicated the presence of two distinct ACPs in *K. aerogenes*, one for the fatty acid synthetase system and the other for its citrate lyase. Tryptic peptide maps of the carboxy-methylated ACPs of citrate lyase from *K. aerogenes* and of the ACPs of the fatty acid synthetases from *E. coli* and *K. aerogenes* showed the absence of any common peptide in the citrate lyase and fatty acid synthetase ACPs (Dimroth, 1975; Bayer and Eggerer, 1978). The ACPs of citrate lyase from *K. aerogenes* and of fatty acid synthetase from *E. coli* have also been shown to have no sequence homologies, except for two very short regions (Beyreuther *et al.*, 1978). Despite this unrelatedness, the two polypeptide chains have been found from CD measurements to have similar  $\alpha$ -helical contents of about 40–50% (Beyreuther *et al.*, 1978).

The ACP subunit of *K. aerogenes* citrate lyase has been shown to contain an acetylated cysteine residue in the polypeptide chain (Dimroth *et al.*, 1973). This has posed the problem of the biologically active acyl carrier in the ACP subunit. Indirect evidence that the prosthetic groups carry the essential acetyl moieties was obtained by affinity labelling of the deacetyl complex with low concentrations of iodoacetate in the presence of an excess of DTE when the cysteamine moiety was exclusively alkylated to yield a carboxymethylated complex inaccessible to reactivation either chemically with acetic anhydride or enzymatically with citrate lyase ligase (Dimroth and Eggerer, 1975b). Direct evidence that the cysteamine residue is the active acyl carrier was obtained when reaction-inactivated citrate lyase was shown to be devoid of acetyl-S-cysteamine while still retaining acetyl-S-cysteine residues (Basu *et al.*, 1983). The cysteamine and cysteine residues of *K. aerogenes* citrate lyase ACP have been shown to be located in close proximity. Oxidation of the deacetylated ACP subunit with Cu<sup>2+</sup>-o-phenanthroline complex led exclusively to intrapeptide bridge formation, indicating that the sulphydryl residues are juxtaposed within 2 Å (Basu *et al.*, 1983). Evidence was also obtained for the involvement of citrate lyase ligase in the post-translational modification of the cysteine residue in the ACP subunit. It was suggested that the acylation of the cysteine residue in the ACP polypeptide chain might be to protect against intrapeptide bridge formation with the closely located sulphydryl of the prosthetic group in the deacetylated enzyme.

**Holoenzyme:** Srere and Singh (1974) and Dimroth and Eggerer (1975b) have discussed a possible mechanism of citrate cleavage by the citrate lyase multienzyme complex. The cleavage mechanism has been postulated to be through the oscillation between the acyl-transferase and acyl-lyase subunits of the prosthetic group on the ACP subunit. An alternate mechanism proposed by Zimmermann *et al.* (1982) postulates the rotation of

the  $\alpha$ -subunit around its vertical axis leading to a transition from the 'ring' form which represents the 'substrate' or citryl-enzyme to the 'star' form whereby the citryl residue on the ACP subunit is assumed to be brought close to the acyl-lyase subunit. On cleavage of the citryl moiety with release of oxaloacetate, the enzyme is assumed to revert to the 'ring' conformation. The latter mechanism is based on electron microscopic observations and cross-linking studies using bifunctional reagents. The working model assumes two layers of subunit molecules positioned one above the other. In each layer, the structural unit is assumed to consist of an  $\alpha$ -subunit with a  $\gamma$ -subunit sitting as a polar cap and a  $\beta$ -subunit. 'Stars' and 'rings' are postulated to be transformed from one to the other by the rotation of the  $\alpha$ -subunit around the vertical axis and opening followed by closing of contacts between  $\beta$ - and  $\gamma$ -component of neighbouring structural units. The 'triangle' form seen in electron micrographs are assumed to be a transition between the 'stars' and 'rings'. Contact with appropriate active sites is assumed to result from the rotation of the  $\alpha$ -subunit.

#### Amino acid composition

The amino acid compositions of the holoenzyme and the separated subunits of *K. aerogenes* citrate lyase have been reported (Bowen and Rogers, 1965; Singh *et al.*, 1976). All the subunits of the enzyme from this source have been shown to have methionine as the amino terminal residue. In the *E. coli* complex, however, the acyl-transferase ( $\alpha$ ) subunit has been shown to have a methionine at the amino terminus while the acyl-lyase ( $\beta$ ) subunit and the unusually large ACP ( $\gamma$ ) subunit have isoleucine at the amino terminus (Nilekani and SivaRaman, 1983).

The amino acid compositions of the  $\alpha$ - and  $\beta$ -subunits of *K. aerogenes* citrate lyase have been reported by Singh *et al.* (1976). The amino acid sequence upto 10 residues from the amino terminus of both subunits have also been reported. An unusual observation has been of microheterogeneity in the  $\beta$ -subunit, equal amounts of proline and arginine occurring at the fourth position from the amino terminus. Amino acid composition of the ACP ( $\gamma$ ) subunit of *K. aerogenes* citrate lyase was reported by Dimroth *et al.* (1973). The sequence analysis of the subunit has been carried out by automated sequential Edman degradations (Beyreuther *et al.*, 1978).

Evidence of the repetitive structure of the unusually large  $M_r$  85,000 ACP ( $\gamma$ ) subunit of *E. coli* citrate lyase has been obtained from the almost quantitative recovery of the components of its four covalently-bound prosthetic groups in a single tryptic fragment. Only a single Pauly-positive fragment could be detected in the tryptic peptide map despite the presence of eight histidine residues in the polypeptide chain. The amino acid composition of the subunit was also characteristic of a protein with a highly repetitive structure. These evidences of sequence homologies in the large ACP subunit of *E. coli* citrate lyase probably reflect intragenic duplications (Subramanian *et al.*, 1984).

#### Nature of citrate lyase prosthetic group

The presence of covalently-bound pantothenate moieties in the holoenzyme from *K. aerogenes* was reported first by Srere *et al.* (1972). Since alkaline phosphatase treatment and alkaline hydrolysis were required for rendering the pantothenate accessible to microbial growth, it was suggested that the prosthetic group probably resembles 4'-

phosphopantetheine of the fatty acid synthetase ACP. It was later recognized that the citrate lyase prosthetic group was more complex and contained adenine, phosphate and sugar in addition to 4'-phosphopantetheine (Dimroth, 1975; 1976). The structure of the prosthetic group was elucidated as (1'' → 2')-(5''-phosphoribosyl)dephospho-CoA from the characterization of several chemical and enzymatic degradation products and from nuclear magnetic resonance and mass spectrometric studies (Robinson *et al.*, 1976; Oppenheimer *et al.*, 1979). The attachment of the prosthetic group to the ACP polypeptide chain was shown to be through serine-14 (Beyreuther *et al.*, 1978). The prosthetic group of citramalate lyase from *C. tetanomorphum* has been shown to be similar to that of *K. aerogenes* (Buckel and Bobi, 1976).

The prosthetic group isolated from *K. aerogenes* citrate lyase after pronase digestion was shown to cross-react with rat liver ATP-citrate lyase, pig heart citrate synthase and HS-citrate lyase ligase (Robinson *et al.*, 1976).

### Immunological behaviour

Citrate lyases from *K. aerogenes*, *E. coli* and *S. diacetylactis* have been shown to be immunologically distinct, cross-reacting only with antisera against the enzyme from the same source (Singh and Srere, 1975). Citrate lyases from the closely related *R. gelatinosa* and *R. palustris*, however, cross-react to a limited extent while being distinct immunologically from *K. aerogenes*, *S. diacetylactis* and *C. sphenoides* citrate lyases (Giffhorn *et al.*, 1981). Only the antibodies against purified  $\alpha$ -subunit of *R. gelatinosa* citrate lyase complex formed precipitin bands with *R. palustris* citrate lyase, while antibodies to  $\beta$ - and  $\gamma$ -subunits showed no cross-reaction. Antibodies against citrate lyase from *C. sphenoides* react with the enzymes from other clostridial species but not with the enzymes from *R. gelatinosa*, *K. aerogenes* and *S. diacetylactis* (Antranikian *et al.*, 1984).

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## Regulation and structure of aspartokinase in the genus *Bacillus*

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**Abstract.** The aspartate pathway of amino acid biosynthesis in bacteria serves as paradigm for the evolution of patterns of enzyme regulation in response to specific physiological requirements. In *Bacillus* species, the first step in the pathway is catalyzed by multiple forms of aspartokinase, which differ in their structure and feedback regulation. One form of aspartokinase (V-type) functions primarily during cell growth, another form (S-type) during sporulation. The V-type aspartokinase from *Bacillus subtilis* and *Bacillus polymyxa* is discussed in some detail on account of its complex pattern of regulation by the pathway endproducts lysine and threonine and its unusual subunit structure. The enzyme is composed of two dissimilar subunits, the smaller of which corresponds to the carboxyl-terminal domain of the larger subunit. The coding sequence for the subunits of *Bacillus subtilis* aspartokinase has recently been cloned in *Escherichia coli*. The study of its structure and mode of expression has revealed that the two aspartokinase subunits are encoded by in-phase overlapping genes. These unusual features of aspartokinase suggest that important aspects of the regulation of the aspartate pathway are yet to be discovered.

**Keywords.** Amino acid biosynthesis; aspartokinase; enzyme regulation; *Bacillus* species; allosteric control; overlapping genes; subunit structure.

## The emergence of molecular cybernetics

It is perhaps fitting to celebrate the Golden Jubilee of the Indian Academy of Sciences with the review of a topic that would have been considered unorthodox, if not unacceptable, at the time of the Academy's founding, and thereby to symbolize by the development of a new area of biochemistry the growth of the scientific enterprise in India. The topic of our discussion is the physiological function of an enzyme and involves viewing enzymes as flexible, adaptable entities whose structures evolved in a specific physiological context. According to this view, not only can function be deduced from structure, but the study of enzyme structure can raise new questions concerning functions yet to be discovered.

This year is also the 50th anniversary of the paper of Lineweaver and Burk (1934), which, by providing a convenient method for measuring the kinetic parameters of enzyme catalysis, contributed materially to the notion of an enzyme as an inflexible engine for the conversion of substrate to product at a rate defined by the parameters  $V_{\max}$  and  $K_m$ . More than 20 years passed until this static picture of enzymes began to be questioned. The impetus for this change in view came from the consideration of the need of bacteria to adapt their metabolism efficiently to a changing environment. When

Umbarger (1956) discovered the inhibition by isoleucine of the first specific enzyme in the pathway for isoleucine biosynthesis, he recognized its functional significance as a feedback control mechanism and went on to predict that all metabolism is controlled according to cybernetic principles. The molecular basis of this kind of feedback control was found by Gerhart and Pardee (1962) to involve binding of the feedback inhibitor to a specific site on the enzyme separate from the catalytic site. The existence of a specific control site implied that enzymes evolved not only to catalyze reactions but to be regulated, a concept that was quickly generalized by Monod *et al.* (1963). This concept of enzymes as allosteric proteins emphasized flexibility both in terms of evolution and structure and carried in it the notion that the physiological function of any particular enzyme is implicit in its properties.

### The aspartate pathway as paradigm

Feedback control by end product inhibition of the first specific enzyme in a biosynthetic pathway is a relatively straightforward matter; more problematical is the control of a pathway that has multiple end products. In order to determine how this problem has been solved by *Escherichia coli*, an organism known for its efficient utilization of nutrients, Stadtman *et al.* (1961) examined the so-called aspartate pathway, a complex biosynthetic pathway for the conversion of L-aspartate to three major end products, the amino acids L-threonine, L-lysine, and L-methionine (figure 1). Not unexpectedly, *E. coli* had evolved an efficient mechanism for controlling the flow of precursors through the aspartate pathway, which involved multiple forms of aspartokinase, the enzyme catalyzing the first step in the pathway, each controlled by one of the pathway end products. Stadtman *et al.* (1961) originally identified two aspartokinases, subject to feedback inhibition by L-lysine and L-threonine respectively; a third aspartokinase under the control by L-methionine was described subsequently (Patte *et al.*, 1967). This type of arrangement assures that each of the end products reduces the flux of the precursor aspartate through the pathway by about one-third, thus still allowing adequate aspartyl-P formation for the synthesis of the other end products. For maximum effectiveness, the enzymes at the pathway branch points must also be subject to feedback regulation, a topic which, together with the detailed investigation of the

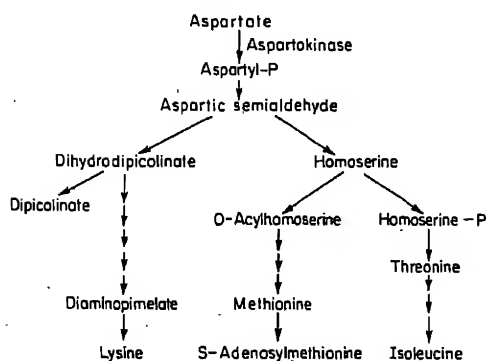


Figure 1. The aspartate biosynthetic pathway.

molecular mechanism of aspartokinase regulation, has been the subject of very elegant studies by G. N. Cohen and coworkers but is beyond the scope of this article.

Are there other way in which branched pathways can be controlled by end products such as to avoid the dilemma of one end product interfering with the production of another? Datta and Gest (1964a) addressed themselves to this question by examining the aspartate pathway in various nonsulphur purple photosynthetic bacteria and discovered an abundance of different control mechanisms. The aspartate biosynthetic pathway thus came to exemplify the diversity of possible solutions to a problem in metabolic regulation. The very existence of such diversity suggested that regulatory mechanisms evolved relatively recently in the context of the specific physiological requirements of an organism. The comparative study of patterns of regulation can thus provide insights into control strategies as well as into the physiological problems that have provoked their evolution.

### The aspartate pathway in *Bacillus* species

In *E. coli*, the aspartate pathway has the relatively simple function of providing amino acids needed for growth; in nongrowing cells, the pathway can be essentially quiescent. A more complex situation obtains in the genus *Bacillus* as a result of its characteristic life cycle. Under adverse nutrient conditions, members of the genus *Bacillus* enter a resting state, the bacterial spore, which can effectively survive prolonged heat, dehydration, and mechanical stress. The process of sporulation involves the synthesis of new structures such as the spore cortex and spore coat; nevertheless, it can occur with a minimum of external nutrients because it involves no net protein and nucleic acid synthesis, the components of new structures being obtained by degradation of existing molecules. It would seem, therefore, that the pathways of amino acid and nucleotide biosynthesis are not needed during sporulation, and many biosynthetic enzymes are indeed specifically degraded at that time. However, an important exception is the aspartate biosynthetic pathway because a major spore component, dipicolinic acid (Powell, 1953), is absent from vegetative cells and must thus be specifically synthesized during sporulation according to the scheme shown in figure 1. An additional demand on the lysine branch of the aspartate pathway during sporulation is the production of large amounts of diaminopimelate for the synthesis of spore cortex mucopeptide. Aspartokinase has thus a dual function in *Bacillus* species: the synthesis of amino acids during growth and the production of dipicolinate and diaminopimelate during sporulation.

Is this dual function reflected in the regulation of aspartokinase? The various forms of aspartokinase that have been described in *Bacillus* species and their regulation are summarized in table 1. The predominant pattern consists of two distinct aspartokinases whose relative amounts differ during vegetative growth and sporulation. The major enzyme in growing cells (V-type) is usually subject to feedback inhibition by lysine or by combinations of lysine and threonine, consistent with the idea that its primary function is to provide amino acids for protein synthesis. The predominant aspartokinase activity during sporulation (S-type) is inhibited by the cell wall mucopeptide constituent meso-diaminopimelate, allowing it to function even in the presence of high levels of protein

Table 1. Aspartokinase activities found in *Bacillus* species.

<i>Bacillus</i> species	Enzyme type	Feedback inhibitors	Subunit structure	Change during sporulation	References
<i>B. subtilis</i> ATCC6051	V	Lysine and threonine	—	Decrease	Rosner and Paulus, 1971
	S	Diaminopimelate	—	Constant	
<i>B. subtilis</i> 60015	V	Lysine	$\alpha_2\beta_2$	Decrease	Hampton <i>et al.</i> , 1971; Vold <i>et al.</i> , 1975;
<i>B. subtilis</i> 168	S	Diaminopimelate	—	Constant	Moir and Paulus, 1977a
<i>B. subtilis</i> VB217	V	Lysine and threonine	—	Constant	Kuramitsu, 1970; Kuramitsu and Yoshimura, 1971; Kuramitsu and Yoshimura, 1972
<i>B. stearothermophilus</i>	S	Diaminopimelate <sup>a</sup>	—	Increase	Forman and Aronson, 1972; Hoganson <i>et al.</i> , 1978
<i>B. cereus</i>	V	Lysine	—	Decrease	Stahly and Bernlohr, 1967; Gray and Bernlohr, 1969
	S	Diaminopimelate	—	Increase	
<i>B. licheniformis</i>	V	Lysine and threonine	—	Decrease	Paulus and Gray, 1967; Biswas <i>et al.</i> , 1970; Nefelova <i>et al.</i> , 1972
	S <sup>b</sup>	None <sup>b</sup>	—	Increase	
<i>B. polymyxa</i>	V	Lysine and threonine	$\alpha_2\beta_2$	—	Ito <i>et al.</i> , 1969
<i>B. colistinus</i>	V	Lysine and threonine	—	—	Hitchcock, 1976; Hitchcock and Hodgson, 1976; Hitchcock <i>et al.</i> , 1980
<i>B. brevis</i>	V	Lysine	$\alpha_2\beta_2$	Decrease	
	V	Lysine and threonine	—	Decrease	
<i>B. megaterium</i>	c	Lysine and methionine	—	(Represented by methionine)	Chatterjee and White, 1982
	c	Threonine	—	(Represented by threonine)	

<sup>a</sup> The effect of diaminopimelate was not tested in *B. subtilis* 60015 (Hampton *et al.*, 1971) and 168 (Vold *et al.*, 1975).

<sup>b</sup> A feedback resistant form of aspartokinase appears during sporulation, but it has not been established whether it is a discrete protein or an altered form of V-type enzyme. Its sensitivity to diaminopimelate has not been tested.

<sup>c</sup> The two forms aspartokinase have not been physically separated.

amino acids to produce the structural components of the spore. To assure the efficient production of dipicolinate and diaminopimelate under these conditions, the lysine branch of the aspartate pathway in *Bacillus* species has two characteristic features. One is the absence of feedback control of dihydrodipicolinase synthase, the first enzyme in the lysine branch (Stahly, 1969; Webster and Lechowich, 1970; Yamakura *et al.*, 1974; Hoganson and Stahly, 1975). This contrasts with the situation in *E. coli*, where this enzyme is specifically inhibited by lysine (Yugari and Gilvarg, 1962), and allows the production of dipicolinate and diaminopimelate even in the presence of lysine. Another is the control of the conversion of diaminopimelate to lysine either by feedback inhibition of diaminopimelate decarboxylase by lysine (Rosner, 1975) or by the inactivation of that enzyme during sporulation (Grandgenett and Stahly, 1971), thereby preventing the needless diversion of diaminopimelate to lysine.

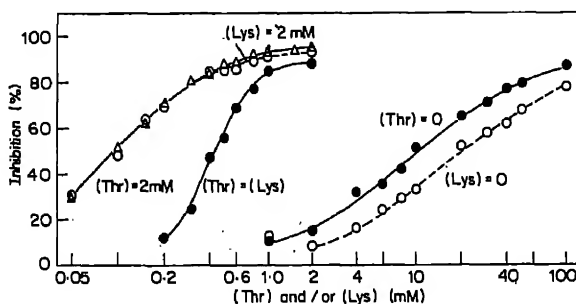
In a few *Bacillus* species, only a V-type aspartokinase has been described (*B. polymyxa*, *B. colistinus*, and *B. licheniformis*). The failure to detect an S-type enzyme does not necessarily indicate its absence but could be due to either the absence of systematic studies under optimal sporulation conditions or the fact that most of these studies were done prior to the discovery of the inhibition of S-type aspartokinase by diaminopimelate (Rosner and Paulus, 1971). Indeed, the aspartokinase activity of *B. licheniformis* has been reported to undergo changes in sensitivity to inhibition by lysine and threonine during the transition from growth to sporulation (Gray and Bernlohr, 1969), which could have been due to the emergence of a separate lysine-insensitive enzyme. To examine the response of that activity to diaminopimelate would be of interest. *Bacillus brevis* is unusual in that it has two separate aspartokinases associated with vegetative growth. Studies on subunit structure have shown that the lysine-sensitive enzyme is homologous to the V-type aspartokinases from *B. subtilis* and *B. polymyxa* but is quite different from the lysine- and threonine-sensitive enzyme produced by the same organism (Hitchcock, 1976). *Bacillus megaterium* also has two aspartokinases, with a pattern of regulation completely different from that seen in any other *Bacillus* species, one enzyme activity being controlled by lysine and methionine, the other by threonine alone (Chatterjee and White, 1982). The possibility that additional aspartokinase activities are present in *B. megaterium* under sporulation conditions has not been examined. The limited diversity seen in the regulation of aspartokinase in *Bacillus* species confirms the notion that regulatory mechanisms are the products of relatively recent evolution in response to specific physiological pressures and may thus differ significantly even in closely related species.

### Allosteric properties of the V-type aspartokinase from *Bacillus polymyxa*

Having discussed aspartokinase in the context of bacterial physiology, let us now examine one type of aspartokinase in greater detail from the point of view of the biochemist and molecular biologist, in order to see whether its properties are consistent with its physiological role or whether they raise new questions concerning metabolic regulation. We will focus on the V-type aspartokinases from *B. polymyxa* and *B. subtilis*, which have been purified to homogeneity and studied in greater detail than other *Bacillus* aspartokinases.



When we first investigated the aspartokinase activity from *B. polymyxa*, the enzyme appeared refractory to inhibition by any of the end products of the aspartate pathway at physiological concentrations (less than 2 mM). However, when the enzyme was exposed to combinations of amino acids, a highly synergistic inhibition by lysine and threonine was observed (Paulus and Gray, 1964). As shown in figure 2, combinations of the two amino acids strongly inhibited aspartokinase activity at concentrations at which neither amino acid alone had an effect on the enzyme (Paulus and Gray, 1967). This phenomenon, which represented a novel mode of regulation of enzyme activity, was discovered independently by Datta and Gest (1964b) in *Rhodopseudomonas capsulata*. It was termed multivalent or concerted feedback inhibition and is now known to be one of the most common modes of control of bacterial aspartokinases. By assuring that moderate concentrations of neither lysine nor threonine alone can completely block aspartokinase activity, multivalent feedback inhibition accomplishes with a single enzyme the same objective as the presence of separate lysine-sensitive and threonine-sensitive aspartokinases in *E. coli* (Stadtman *et al.*, 1961). On the other hand, although multivalent feedback inhibition of aspartokinase by lysine and threonine assures that these amino acids do not interfere with each other's synthesis, this mode of regulation can be detrimental due to limitation of methionine biosynthesis by lysine and threonine (Burlant *et al.*, 1965). It is possible, however, that this defect is revealed only under the contrived conditions of the laboratory, situations in nature where high concentrations of lysine and threonine occur in the absence of other amino acids probably being rare.



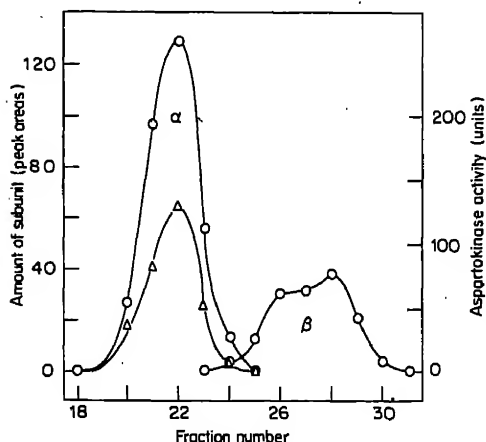
**Figure 2.** Inhibition of the V-type aspartokinase of *B. polymyxa* by lysine and threonine. The curves, from left to right, represent experiments with varying L-lysine at 2 mM L-threonine, varying L-threonine at 2 mM L-lysine, varying L-lysine and L-threonine at equimolar concentrations, varying L-lysine alone and varying L-threonine alone (from Paulus and Gray, 1967).

Another aspect of aspartokinase regulation deserves comment because it is still problematical. Early studies on the aspartokinase from *B. polymyxa* revealed that its activity was modulated not only by lysine and threonine but also by any of a large variety of nonpolar L-amino acids (Paulus and Gray, 1968). A detailed analysis of the effects of nonpolar amino acids on aspartokinase was consistent with a specific binding site, but the fact that various nonpolar amino acids could either counteract or enhance the inhibition by lysine and threonine made it difficult to visualize a physiological

function. Nevertheless, it is of interest that aspartokinases from a number of other bacterial species are also affected by nonpolar amino acids. Whereas the predominant effect of nonpolar amino acids on the *B. polymyxa* aspartokinase is activation, nonpolar L-amino acids strongly potentiate the inhibition of *E. coli* aspartokinase III by lysine (Patte *et al.*, 1965). Stimulation of aspartokinase activity has also been observed in *Myxococcus xanthus* (Filer *et al.*, 1973), *B. polymyxa* Ross (Nefelova *et al.*, 1972), and *B. subtilis* ATCC 6051 (Rosner and Paulus, 1971), but not in *B. subtilis* VB217 (Moir and Paulus, 1977a). The ability of many different aspartokinases to interact with nonpolar amino acids suggests the existence on the enzyme of a binding site that has persisted through the course of evolution but has diverged as far as the effect of the binding on enzyme activity is concerned. Whether this site represents an evolutionary vestige or has a present-day regulatory function, perhaps in the integration of different pathways of amino acid biosynthesis, remains to be elucidated.

### Subunit structure of the V-type aspartokinase

In their classical paper, Monod *et al.* (1965) argued for a causal relationship between the quaternary structure and allosteric regulation of an enzyme. According to that view, the study of subunit structure should promote a deeper understanding of enzyme regulation. It was thus of considerable interest that the aspartokinase of *B. polymyxa* had an unusual subunit structure, being composed of two dissimilar subunits,  $\alpha$  and  $\beta$ , with molecular weights of 43,000 and 17,000, respectively, and the composition  $\alpha_2\beta_2$  (Biswas *et al.*, 1970). A similar structure was later found for the V-type aspartokinase of *B. subtilis* (Moir and Paulus, 1977a) and one of the aspartokinases of *B. brevis* (Hitchcock, 1976). The analysis of the cross-linked products obtained after treatment with the bifunctional reagent dimethyl suberimidate showed the aspartokinase of *B. polymyxa* to consist of a dimer of  $\alpha$ -subunits flanked by  $\beta$ -subunits, thus being defined by one  $\alpha$ - $\alpha$  and two  $\alpha$ - $\beta$  bonding domains, with no  $\beta$ - $\beta$  interactions (Biswas and Paulus, 1973). The subunits could be reversibly dissociated by 4 M urea and separated by gel filtration on Sephadex G-200. As shown in figure 3, upon removal of urea by



**Figure 3.** Separation of the subunits of the V-type aspartokinase of *B. polymyxa* by gel filtration on Sephadex G-200 in the presence of 4 M urea. Amounts of subunit (O) were estimated from staining intensity on polyacrylamide gels. Aspartokinase activity ( $\Delta$ ) was measured after dilution of urea to 80 mM (from Biswas and Paulus, 1973).

dialysis, aspartokinase activity was regained in the fractions containing only  $\alpha$ -subunit but not in the  $\beta$ -subunit fractions (Biswas and Paulus, 1973). Further analysis of the enzyme activity thus recovered revealed that renatured  $\alpha$ -subunit had all the catalytic and allosteric properties characteristic of the native enzyme. This observation was most surprising, as it implied that the  $\beta$ -subunit of aspartokinase contributed neither to catalysis nor to the regulation of the enzyme. Equally puzzling was the association behavior of the separated aspartokinase subunits upon renaturation. Analysis by polyacrylamide gel electrophoresis and cross-linking studies revealed that renatured  $\alpha$ -subunit consisted primarily of dimers, tetramers, and hexamers, whereas  $\beta$ -subunit existed only as dimers. On the other hand, renaturation of equimolar mixtures of  $\alpha$ - and  $\beta$ -subunits yielded only an  $\alpha_2\beta_2$  species characteristic of the native enzyme (figure 4). These observations implied that the  $\alpha$ - $\beta$  bonding domain had an element of symmetry so that the regions involved in the interaction of  $\alpha$ - and  $\beta$ -subunits would also have a tendency to self associate.

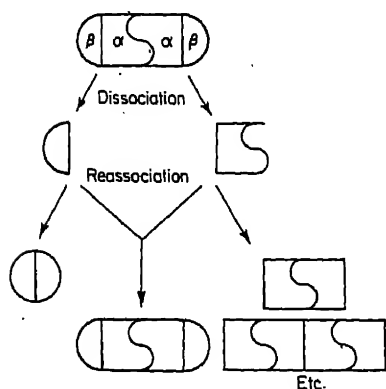


Figure 4. Model for the dissociation and reassociation of aspartokinase subunits.

In order to clarify these aspects, we turned to the V-type aspartokinase of *B. subtilis* VB217, a strain genetically derepressed for aspartokinase synthesis and thus a convenient source of large amounts of enzyme (Yeh and Steinberg, 1978). Unlike the aspartokinase from *B. polymyxa*, the V-type enzyme of *B. subtilis* VB217 is not subject to multivalent feedback inhibition but is inhibited by lysine alone. However, in all other respects, including subunit structure and arrangement and behavior upon subunit dissociation and renaturation, the *B. subtilis* V-type aspartokinase was indistinguishable from the *B. polymyxa* enzyme (Moir and Paulus, 1977a). The comparison of the  $\alpha$ - and  $\beta$ -subunits of the *B. subtilis* aspartokinase revealed an interesting relationship. Double immunodiffusion experiments with antibodies raised against the smaller  $\beta$ -subunit revealed a reaction of identity between the aspartokinase subunits (figure 5), while similar experiments with anti- $\alpha$ -subunit antibodies revealed a reaction of partial identity, suggesting that the antigenic determinants of the  $\beta$ -subunit constituted a subset of the  $\alpha$ -subunit determinants (Moir and Paulus, 1977b). This notion was extended to the amino acid sequence of the subunit by comparing the products of trypsin hydrolysis of the two subunits. As shown in figure 6, all but one of the tryptic peptides derived from  $\beta$ -subunit were identical with  $\alpha$ -subunit peptides, indicating that

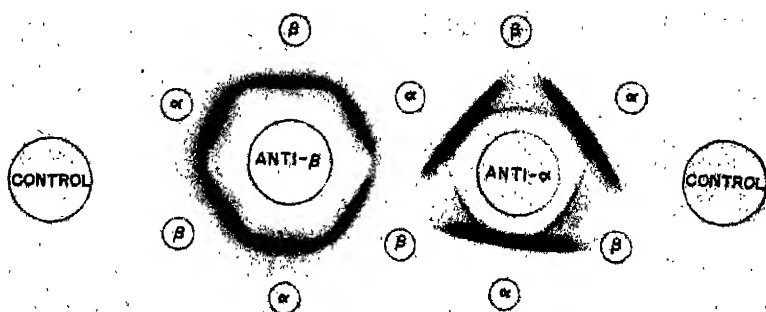


Figure 5. Double immunodiffusion of the purified subunits of the *B. subtilis* V-type aspartokinase against anti- $\alpha$ , anti- $\beta$ , and control sera (from Moir and Paulus, 1977b).

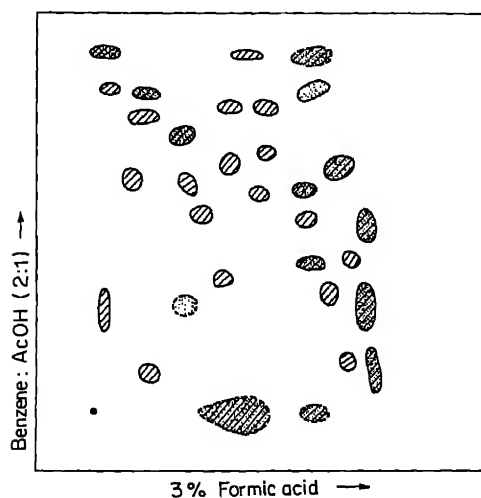


Figure 6. Map of the tryptic peptides derived from the  $\alpha$ - and  $\beta$ -subunits of *B. subtilis* V-type aspartokinase. A mixture of 150 pmol of dansylated  $\alpha$ -peptides and 5 pmol of [ $^3\text{H}$ ]-dansylated  $\beta$ -peptides were subjected to two-dimensional chromatography on polyamide sheets in the solvents indicated. Peptides derived from  $\alpha$ -subunit, detected by fluorescence, are indicated by cross-hatching; peptides derived from  $\beta$ -subunit, detected by fluorography, are indicated by stippling; spots of non-peptide origin are indicated by dashed borders (from Moir and Paulus, 1977b).

the amino acid sequence of  $\beta$ -subunit was contained within the larger  $\alpha$ -subunit. By determining the amino- and carboxyl-terminal amino acid residues of the two subunits, their relationship could be defined as illustrated in figure 7, which shows that the  $\beta$ -subunit corresponds to the carboxyl-terminal segment of the  $\alpha$ -subunit.

The observation that the two aspartokinase subunits had an amino acid sequence in common helped to explain the unexpected reassociation properties summarized earlier in figure 4. In order to explain these properties, it was necessary to assume that the  $\alpha$ - $\beta$  domain of bonding had an element of symmetry. The molecular basis for this symmetry



Figure 7. Relationship between the structure of the  $\alpha$ - and  $\beta$ -subunits of the V-type aspartokinase from *B. subtilis*.

could now be understood in terms of the sequence homology between the aspartokinase subunits, the  $\alpha$ - $\beta$  bonding domain actually being the vestige of a second type of  $\alpha$ - $\alpha$  interaction (figure 8). The common bonding surface on the two subunits, designated  $b$ - $b'$ , will not only permit the interaction between  $\alpha$ - and  $\beta$ -subunits to form the  $\alpha_2\beta_2$  structure but also interaction between  $\alpha$  dimers to form higher multiples such as tetramers and hexamers as well as interaction between  $\beta$  monomers to form dimers.

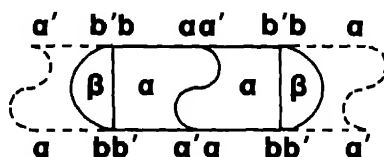
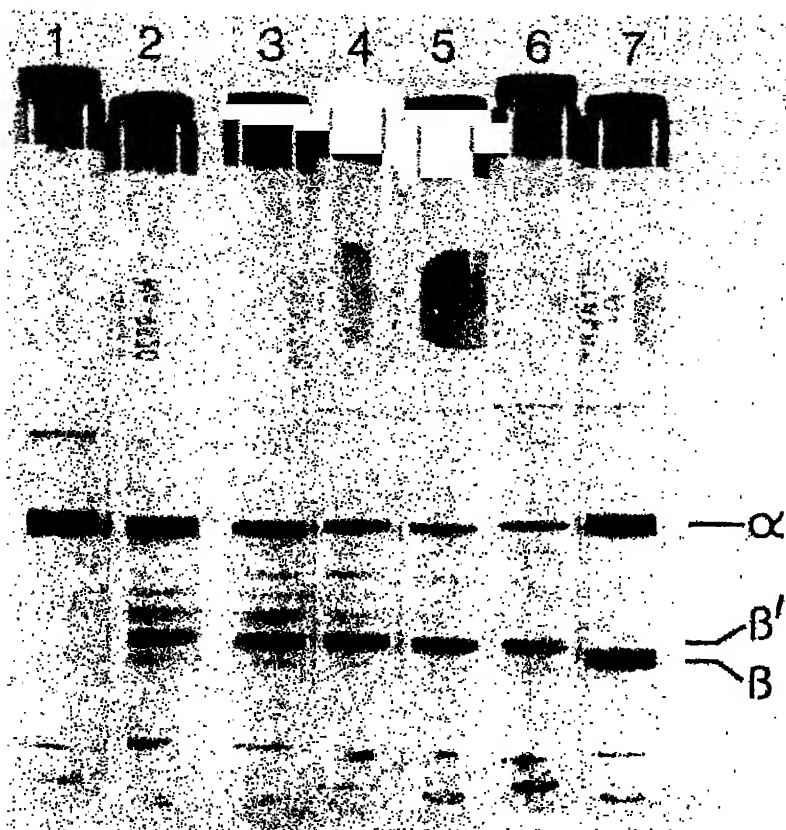


Figure 8. Model for subunit interactions in the *B. subtilis* V-type aspartokinase. The dashed outline indicates the structural relationship between  $\alpha$ - and  $\beta$ -subunits.

The relationship between  $\alpha$ - and  $\beta$ -subunits in terms of 3-dimensional structure was examined by subjecting purified  $\alpha$ -subunit, primarily a tetramer, to hydrolysis by trypsin (Bondaryk, 1984). Under these conditions, a polypeptide of molecular weight slightly larger than  $\beta$ -subunit ( $M_r = 19,000$ ) appeared rapidly and remained as a major product, while  $\alpha$ -subunit showed a corresponding decline but then reached a limiting level, which changed little even after prolonged exposure to trypsin (figure 9). The initial rapid degradation to a  $\beta$ -subunit-like material, which was not further degraded, indicated the presence in  $\alpha$ -subunit of a compact globular domain relatively resistant to proteolysis. On the other hand, the failure of  $\alpha$ -subunit to be degraded completely, a substantial fraction remaining as a trypsin-resistant core, suggests the presence of two classes of  $\alpha$ -subunits. A possible interpretation of these observations is suggested by figure 8, in which the dotted outline depicts an  $\alpha$ -subunit tetramer, arranged as a dimer of dimers such that the  $\alpha$ -subunits constitute two non-equivalent sets. The peripheral  $\alpha$ -subunits are degraded by trypsin to a protease-resistant globular domain, the  $\beta$ -subunit, whereas the central  $\alpha$ -subunits are not attacked by the protease. Selective proteolysis has been used to reveal functional domains in a number of other enzymes, for example the bifunctional aspartokinase I-homoserine dehydrogenase I of *E. coli* (Veron *et al.*, 1972). Our results suggest that the  $\beta$ -subunit of the V-type aspartokinase from *B. subtilis* corresponds to a discrete functional domain of the  $\alpha$ -subunit. However, since the  $\beta$ -subunit carries neither the catalytic nor regulatory sites of the enzyme (Moir and Paulus, 1977a), its function remains to be defined.

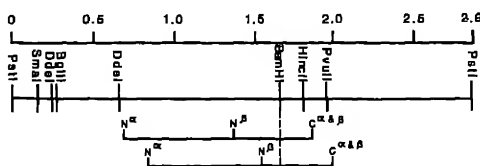


**Figure 9.** Time course of the digestion of the V-type aspartokinase from *B. subtilis* by trypsin. Purified  $\alpha$ -subunit (90  $\mu$ g) was incubated with trypsin-Sepharose (equivalent to 1.4  $\mu$ g trypsin) at 0°. Samples were removed at 0 h (gel 1), 0.5 h (gel 2), 1 h (gel 3), 2 h (gel 4), 4 h (gel 5), and 80 h (gel 6), and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. For comparison,  $\alpha$ - and  $\beta$ -subunits are shown (gel 7) (from Bondaryk, 1984).

### Molecular cloning of the V-type aspartokinase gene of *B. subtilis*

At this stage in the study of the V-type aspartokinase of *B. subtilis*, central questions concerning the function of the  $\alpha$ - and  $\beta$ -subunits and their biosynthetic relationships had arisen whose answers were not easily accessible through standard biochemical approaches. New insights were needed, and we felt that these might be provided by an understanding of the nature of the genetic coding sequence of aspartokinase. We therefore undertook to search for the aspartokinase gene in a *B. subtilis* genomic library carried in a  $\lambda$  charon vector (Ferrari *et al.*, 1981) by an immunological screening procedure. Upon examining the products from more than 5,000 bacteriophage plaques, we succeeded in identifying two recombinant phage lysates that produced antigenic material related to *B. subtilis* aspartokinase (Bondaryk, R. and Paulus, H., 1984, unpublished observations). These carried an 18-kilobase *B. subtilis* genomic fragment,

which could be subdivided into smaller fragments by cleavage with *EcoRI* endonuclease and introduced into the plasmid *pBR322*. Introduction of a recombinant plasmid carrying a 5.8 kilobase *B. subtilis* insert into *E. coli* gave rise to transformant colonies that produced both aspartokinase subunits, and similar results were obtained by an even smaller subfragment (2.9 kilobases) produced with *PstI* endonuclease. In order to define the coding region for the aspartokinase subunits, this relatively small DNA segment was subdivided by cleavage with a variety of restriction endonucleases. A most striking result was obtained when a fragment produced by *BamHI* endonuclease was reinserted into *pBR322* and introduced into *E. coli*. The resulting transformed strain produced antigenic material related to aspartokinase, but molecular weight analysis revealed aspartokinase subunits of 40,000 and 14,000 molecular weight, each 3,000 less than the normal aspartokinase subunits (43,000 and 17,000, respectively). In other words, a single genetic change, produced by deletion of a DNA segment, affected both aspartokinase subunits in an equivalent manner. This observation constituted a strong evidence that the two aspartokinase subunits were encoded by a single gene. Moreover, it was possible to define the polarity and the approximate position of this gene in relationship to other restriction endonuclease sites on the 2.9 kilobase *PstI* fragment. Figure 10 shows the two possible positions of the aspartokinase gene, deduced from the locations of two polypeptide chain termination codons in *pBR322* upstream from the *BamHI* site and from the size of the polypeptides produced in cells transformed with plasmids carrying the *BamHI* cleavage product of the aspartokinase gene.



**Figure 10.** Map of the DNA fragment encoding the V-type aspartokinase from *B. subtilis*. The positions of various restriction endonuclease sites are shown as well as the possible location of the aspartokinase coding region in two possible reading frames defined by termination codons at positions 493 and 624 of *pBR322* (Sutcliffe, 1979). The possible translation initiation sites for  $\alpha$ -subunit ( $N^\alpha$ ) and  $\beta$ -subunit ( $N^\beta$ ) as well as the translation termination site for both subunits ( $C^{\alpha, \beta}$ ) are also shown. The numerical scale indicates DNA length in kilobases.

### Expression of the *B. subtilis* aspartokinase gene in *E. coli*

That the single gene for the two aspartokinase subunits defined by this analysis indeed carried all information for the synthesis and functional expression of the enzyme was confirmed by introducing the recombinant plasmid carrying the 2.9 kilobase *PstI* fragment into an *E. coli* strain devoid of aspartokinase. *E. coli* GIF106M1 lacks all three aspartokinase activities characteristic of *E. coli* and thus requires for growth the three major end products of the pathway, lysine, methionine, and threonine (Theze *et al.*, 1974). When transformed with a plasmid carrying the *B. subtilis* aspartokinase gene,

is strain gained the ability to grow without amino acid supplements, an indication that the *B. subtilis* aspartokinase gene was functionally expressed and could effectively substitute for the *E. coli* aspartokinases. A plasmid carrying only a portion of the aspartokinase gene due to cleavage by *Bam*HI endonuclease could not suppress the *E. coli* aspartokinase lesion, demonstrating that this effect required the intact aspartokinase coding sequence and not merely an accessory genetic element.

An interesting question was whether the *B. subtilis* aspartokinase activity expressed in the transformed *E. coli* strain was responsive to feedback regulation. If the enzyme was subject to feedback inhibition by L-lysine as in *B. subtilis*, one would expect the addition of lysine to be growth inhibitory to the transformed strain by interfering with the synthesis of threonine and methionine. Such a response was indeed observed; moreover, the growth inhibition of transformed *E. coli* GIF106M1 by lysine could be reversed by the addition of homoserine, confirming that it was due to inhibition of threonine and methionine biosynthesis as predicted (Bondaryk, R. and Paulus, H., 1984, unpublished observations). This is the first example of a recombinant gene not only functioning in a foreign host but responding normally to an allosteric control mechanism.

Further evidence that the properties of the aspartokinase expressed in *E. coli* GIF106M1 under the direction of the *B. subtilis* gene were identical to those of *B. subtilis* aspartokinase II came from the study of the allosteric properties of aspartokinase in cell-free extracts of the transformed host. The levels of aspartokinase activity in such extracts were more than 10 times higher than in the *B. subtilis* wild type strain GSY225 and about one-half that of the derepressed derivative *B. subtilis* VB217 (Leh and Steinberg, 1978), and the aspartokinase activity induced in *E. coli* by the recombinant plasmid showed a similar sensitivity to feedback inhibition by L-lysine as the enzyme purified from *B. subtilis* (Bondaryk, R. and Paulus, H., 1984, unpublished observations).

### biosynthetic origin of the aspartokinase subunits

The discovery that a single gene encodes both subunits of the V-type aspartokinase of *B. subtilis* provides no definitive answer to the question of their biosynthetic relationship, being consistent either with the post-translational processing of a single precursor polypeptide or with independent translation of the two subunits. Attempts had been made earlier to evaluate the first possibility by pulse-chase experiments both in *B. subtilis* and in *E. coli* transformed with the aspartokinase gene, but no evidence for precursor-product relationship between the aspartokinase subunits emerged from these studies (Bondaryk, 1984). On the other hand, if the two aspartokinase subunits are translated independently, one might expect to see large effects on the relative rates of  $\alpha$ - and  $\beta$ -subunit synthesis when the steady state synthesis of mRNA was interrupted. This was tested in *E. coli*, transformed with a recombinant plasmid carrying the aspartokinase gene, by inhibiting the synthesis of new mRNA with rifampicin and measuring the rates of  $\alpha$ - and  $\beta$ -subunits synthesis by pulse-labelling with [ $^{35}$ S]-methionine at various times after rifampicin addition (Bondaryk, R. and Paulus, H., 1984, unpublished observations). As shown in table 2, the ability to synthesize  $\alpha$ -



Table 2. Pulse labelling of the aspartokinase subunits at various times after the addition of rifampicin in *E. coli* carrying the *B. subtilis* V-type aspartokinase gene.

Time between rifampicin and [ <sup>35</sup> S]-methionine addition (min)	Radioactivity in $\alpha$ -subunit (cpm)	Radioactivity in $\beta$ -subunit (cpm)	Labelling ratio $\beta/\alpha$ (molar ratio)
Control (no rifampicin)	2755	3287	3.0
1	517	2039	9.9
5	118	713	15.1
15	34	203	14.9

Exponentially growing cells were treated with rifampicin (200  $\mu\text{g/ml}$ ) and at various times thereafter, samples were removed, labelled with [<sup>35</sup>S]-methionine for 10 min, and the aspartokinase subunits were isolated by indirect immunoabsorption and polyacrylamide gel electrophoresis. A sample of a control culture without rifampicin was also labelled. The molar labelling ratios were calculated on the basis of the known amino acid composition of the aspartokinase II subunits (Moir and Paulus, 1977b).

subunit decreased much more rapidly than the ability to synthesize  $\beta$ , so that the relative rates of synthesis of the two subunits changed more than 5-fold over a 15-min period. This suggested that the mRNA from which  $\alpha$ -subunit is translated is less stable than functional  $\beta$ -subunit mRNA. An alternate possibility, that rifampicin causes the selective degradation of  $\alpha$ -subunit, was ruled out by appropriate pulse-chase experiments in the presence of the drug. Indeed, the finding that  $\beta$ -subunit could be synthesized in the virtual absence of  $\alpha$ -subunit synthesis (see the 15-min point in table 2) clearly indicated that translation of  $\alpha$  and  $\beta$ -subunits were independent processes and conclusively eliminated the possibility that  $\beta$ -subunit was post-translationally derived from  $\alpha$ -subunit. Rather, the observation that  $\beta$ -subunit synthesis was not dependent on  $\alpha$ -subunit synthesis supported the view that the two aspartokinase subunits are translated separately from the same DNA sequence in the same reading frame but starting at different translation initiation sites. In other words, the subunits of the V-type aspartokinase of *B. subtilis* are encoded by an in-phase overlapping gene system.

The observation that the  $\alpha$ - and  $\beta$ -subunits of *B. subtilis* aspartokinase are encoded by overlapping genes is of great interest. Overlapping gene systems had previously been observed on the genomes of bacterial and animal viruses (Normark *et al.*, 1983) and had been thought to have evolved as a means for packing a maximum amount of genetic information into a genome of limited size. Such a rationale is not relevant to the bacterial chromosome, which has no stringent size limitation, and indeed only one example of bacterial overlapping genes, the *cheA* locus of *E. coli*, has been described (Smith and Parkinson, 1980). Since the product of the *cheA* gene has not been identified, it has not been possible to suggest alternative functions for this type of genetic arrangement. With aspartokinase, we have the first example of bacterial overlapping genes encoding well-characterized polypeptides whose biochemical relationship is understood. In-phase overlapping genes will automatically generate the type of structural relationship that we have identified between the aspartokinase subunits (figures 7 and 8), and this may well be a major function of such a genetic arrangement. Indeed, in one other case of overlapping genes, those encoding the C and Nu3 proteins of bacteriophage  $\lambda$ , a direct interaction of the gene products has been described (Shaw and Murialdo, 1980), but in a number of other cases, protein-protein

interactions between the products of overlapping genes have been suggested. The V-type aspartokinase of *B. subtilis* may thus represent an especially clear example of what may be the major functional rationale for the evolution of in-phase overlapping gene systems.

It will be of interest to reexamine the function of the  $\beta$ -subunit of aspartokinase in the light of this new information, especially since the realization that the aspartokinase subunits are translated independently from overlapping genes provides us with an effective experimental tool for such an investigation. Upon completion of the nucleotide sequence analysis of the aspartokinase gene, it should be a relatively straightforward matter, through site-directed mutagenesis, to block specifically the translation initiation of  $\beta$ -subunit without interfering with the through-translation of  $\alpha$ -subunit. The examination of the phenotype of bacterial strains that have thus selectively lost the ability to produce the smaller aspartokinase subunit should bring us a great deal closer to the elucidation of its function.

### Prospect

With the discovery of multivalent feedback inhibition of aspartokinase by lysine and threonine, we thought that we had made considerable progress in understanding the regulation of the aspartate pathway in *Bacillus* species. In the 20 years which followed, that goal continuously receded as progress was added to progress, such as the discovery of the diaminopimelate-sensitive aspartokinase, the elucidation of aspartokinase subunit structure, the clarification of the structural basis of aspartokinase subunit interaction, and finally the cloning and characterization of the aspartokinase gene. This apparently retrograde motion was not due to a failure to answer questions but to our ability to ask more sophisticated and deeper ones. At this very moment we are in the position to inquire into the meaning of gene structure in terms of protein interactions and we have in our hands, through directed mutagenesis, the tools to subject our ideas to experimental test. If we are fortunate, these inquiries will allow us to ask new questions that could not have been asked 20 years ago.

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## Conformational change of 23S RNA in 50S ribosome is responsible for translocation in protein synthesis

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**Abstract.** Since the recognition of the 'translocation' phenomenon during protein synthesis several theories have been proposed, without much success, to explain the translocation of peptidyl tRNA from the aminoacyl site to the peptidyl site. The involvement of L7/L12 proteins and therefore the L7/L12 stalk region of 50S ribosomes in the translocation process has been widely accepted. The mobility of the stalk region, as recognised by many workers, must be of physiological significance. It has recently been shown in this laboratory that 50S ribosomes derived from tight and loose couple 70S ribosomes differ markedly in quite a few physical and biological properties and it appears that these differences are due to the different conformations of 23S RNAs. It has also been possible to interconvert tight and loose couple 50S ribosomes with the help of the agents, elongation factor -G, GTP (and its analogues) which are responsible for translocation. Thus loose couple 70S ribosomes so long thought to be inactive ribosomes are actually products of translocation. Further, the conformational change of 23S RNA appears to be responsible for the interconversion of tight and loose couple 50S ribosomes and thus the process of translocation. A model has been proposed for translocation on the basis of the direct experimental evidences obtained in this laboratory.

**Keywords.** Ribosomes; translocation; protein synthesis; ribosomal RNA.

### Introduction

During protein synthesis the peptide bond is formed by the transfer of the peptidyl moiety of the peptidyl tRNA attached to the ribosome at the *P* site to the aminoacyl moiety of tRNA attached to the *A* site (Brot, 1977). This leads to the elongation of the polypeptidyl chain by one amino acid and the former becomes associated with the tRNA at the *A* site. At the next step the peptidyl tRNA is translocated from the *A* site to the *P* site (figure 1). Apparently this translocation results in the movement of mRNA due to locking with tRNA through codon-anticodon interaction. The translocation is effected by the elongation factor-G (EF-G) and GTP during the association with 50S ribosome (Kaziro, 1978; Weissbach, 1980). Proteins L7/L12 which are quite distinct from other ribosomal proteins in many respects, have been implicated in the elongation process (Brot and Weissbach, 1981) although there is some

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Abbreviation used: EF-G, Elongation factor-G.

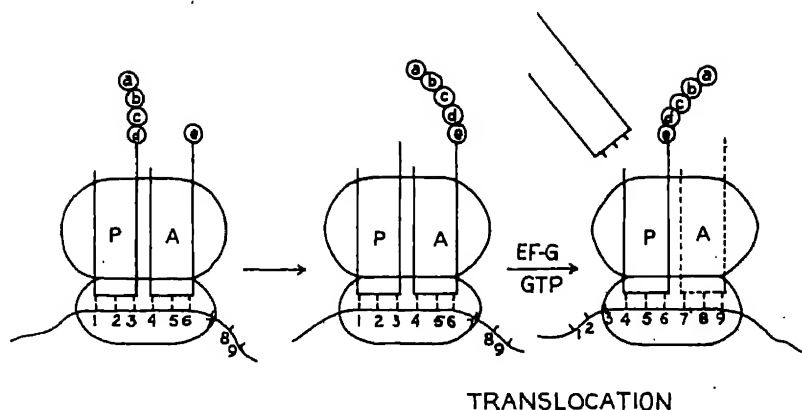


Figure 1. Translocation in protein synthesis.

controversy about this (Liljas, 1982). Similarly EF-G-dependent hydrolysis of GTP as catalysed by ribosome is thought to be connected with translocation although it is not unequivocally agreed upon (Kaziro, 1978; Nierhaus, 1982). GTPase activity is, however, found to be dependent on L7/L12 (Lockwood *et al.*, 1974).

Several mechanisms have been proposed for the translocation process. One of the earlier models, the locking and unlocking ribosome of Spirin (1969) assumes that 70S ribosomes can exist in two different states, locked (with the 30S and 50S subunits closely associated) and unlocked (with the 30S and 50S subunits drawn somewhat apart). The two subunits are envisaged as being hinged to each other and the locking and unlocking of the subunits of the ribosomes are assumed to provide the driving mechanism for translocation. According to the inchworm theory of Hardesty *et al.* (1969), a 'kink' is created in the mRNA during the enzymatic binding of aminoacyl tRNA to the A site of ribosome. EF-G and GTP are assumed to straighten out the 'kink' and in doing so peptidyl tRNA is translocated from the A site to the P site of ribosome. Leder's (1973) model is based on the substrate-product relationship for an enzyme. The weakness of this hypothesis has been discussed in detail by Nierhaus (1982). The latter has proposed a new hypothesis arguing that there is a third site, 'exit site' (E site) for the binding of aminoacyl tRNA. None of these models, however, provide satisfactory explanation of the translocation process itself and each one lacks strong experimental support. In this connection the nonenzymatic translocation *i.e.*, translocation in the absence of EF-G, reported by Pestka (1969) and Gavrilova and Spirin (1971) is quite intriguing. However, the efficiency of such a system is only 1% of the enzymatic one. It may not be out of place to mention here that Woese (1970) suggested a model as an alternate to the translocation from A to P site. It involves the conformational change of tRNA. Similar conformational change of tRNA has been implicated by Lake (1980) to explain the switch over of tRNA from R (recognition) site to aminoacyl site, however, without resulting in translocation. Although the mechanism of translocation still remains obscure experimental evidences will be summarised here to indicate that the conformational change of 23S RNA is responsible for translocation.

### Involvement of L7/L12 stalk region in translocation

As already mentioned in the 'introduction', L7/L12 proteins are involved in translocation (Brot and Weissbach, 1981). No GTPase activity is observed in the absence of these proteins. There was some controversy about the location of L7/L12 (Liljas, 1982) but Strycharz *et al.* (1978) unequivocally demonstrated that these are located at a region known as 'stalk' region, protruding out of the main body. All the 4 copies of L7/L12 are thought to be located in this region and bound to 23S RNA through the protein L10 (Schrier *et al.*, 1973; Dijk *et al.*, 1979; Pettersen 1979; Tokimatsu *et al.*, 1981). They are bound to L10 through N-terminals and their C-terminals protrude out. However, it is quite interesting to note that proton magnetic resonance studies indicated an exceptional mobility of L7/L12 in the ribosome (Gudkov *et al.*, 1972) and supported the proposal of L7/L12 being a dimeric motile protein (Kischa *et al.*, 1971). ESR studies also indicated that L7/L12 might be a flexible domain of the 70S ribosome (Tritton, 1978). Further, Moller *et al.* (1983) have proposed from experimental data that each 50S ribosomal particle possesses two binding sites, each site being involved in the binding of one dimer of L7/L12. Binding of L7/L12 dimer at one site gives rise to the formation of the L7/L12 stalk whereas the binding at the other site has no effect on the number of visible stalks. Crosslinking experiments of Traut *et al.* (1983) also indicate that the L7/L12 stalk may not be a static structure. Conformational changes in L7/L12 upon binding of tRNA to the ribosomes have been suggested by Lee *et al.* (1981). These observations have naturally led to the speculation that the mobility of L7/L12 stalk may be connected with the translocation process.

Extensive work has been carried out in this laboratory on the structure of ribosomal subunits with the help of the single strand-specific enzyme RNase I (Burma, 1979a,b, 1982, 1984a,b; Burma *et al.*, 1980). The 30S ribosome is highly resistant to the action of RNase I in presence of  $Mg^{2+}$  at a concentration of 1 mM or so whereas 50S subunit is quite susceptible under such condition. In the presence of high  $Mg^{2+}$  concentrations (20 mM or so) 50S ribosomes also become resistant but only after L7/L12 and L10 proteins (along with L4) are removed (Raziuddin *et al.*, 1979) and 23S RNA is split in the stalk region (A. K. Srivastava, unpublished results). Subsequently it was shown that 50S ribosomes become resistant to RNase I when L7/L12 proteins are removed by treatment with 1 M  $NH_4Cl$  and ethanol (Byasmuni and Burma, 1982) indicating thereby that L7/L12 proteins unfold the structure of 23S RNA in the stalk region. Recently two populations of 50S ribosomes have been isolated (to be discussed later), one population being highly sensitive to RNase I whereas the other one, highly resistant to the enzyme (Burma *et al.*, 1984b). These data clearly indicate that some part(s) of 23S RNA (most probably in the stalk region) is capable of assuming two conformations, one folded and the other one, having open structure, highly susceptible to the action of RNase I. This will be discussed in detail later.

### Tight and loose couple ribosomes and translocation

It is well known that there are two types of 70S ribosomal populations, the tight couples which remain associated at low  $Mg^{2+}$  concentrations (4 mM or so) and loose couples

which require higher  $Mg^{2+}$  concentrations (10 mM or so) for association. It has been suggested that the ribosomes which dissociate readily (loose couples) may be damaged ones whereas tight couples are active particles (Noll *et al.*, 1973a,b; Hapke and Noll, 1976). There were a number of reports suggesting that the ribosomes undergo conformational change during translocation (Schrier and Noll, 1971; Chuang and Simpson, 1971; Waterson *et al.*, 1972). The suggestion was made on the basis of the observed differences in the sedimentation values of pre- and post-translocation ribosomal complexes during high speed centrifugation. The interpretation of the results was, however, questioned since this could be an artifact of pressure-induced dissociation (Infante and Baherlein, 1971; Infante and Krauss, 1971). However, Van Diggelen *et al.* (1971) showed that the ability to form the two association products is the intrinsic property of 50S subunits. These workers studied the association properties of 'native' subunits (which are present in the free state in the extract) and 'derived' subunits which are obtained by the dissociation of 70S ribosomes. It is most likely that the derived subunits are 50S ribosomes present in tight couples and native subunits are components of loose couples. No difference in the constituents of the two populations of 50S ribosomes was, however, detected although these workers (Van Diggelen and Bosch, 1973; Van Diggelen *et al.*, 1973) strongly felt that the two types of ribosomes differ in a ribosomal protein.

It has been shown in this laboratory that there is specific interaction between 16S and 23S RNAs which form the bimolecular complex under two well-defined conditions (Nag and Burma, 1982; Burma *et al.*, 1983; Nag *et al.*, 1984). In order to demonstrate that such association reflects the mechanism of association of 30S and 50S subunits, the method of kethoxal treatment, as extensively used by Noller and his coworkers (Herr and Noller, 1978, 1979; Herr *et al.*, 1979) for the modification of ribosomes, was followed (Nag and Burma, 1984). As shown by Noller and his coworkers, 30S ribosomes on treatment with kethoxal completely lose their capacity to associate with 50S ribosomes. Surprisingly enough, 16S RNA isolated from kethoxal-treated 30S ribosomes or directly treated with kethoxal also loses its capacity to associate with 23S RNA. However, on treatment of 50S ribosomes with kethoxal only half of the population lost their capacity to associate with 30S ribosomes. This was at variance with the observation of Herr and Noller (1979) who observed complete loss. Similarly, only half of the population of 23S RNA either derived from kethoxal-treated 50S subunits or directly treated with kethoxal lost their capacity to associate with 16S RNA. These results indicated that there are two distinct populations of 50S ribosomes which perhaps originate from the two populations of 23S RNA. But the most important observation is that the two RNAs have quite different conformations and thus behave differently to kethoxal treatment.

As mentioned above, the results obtained with the 50S ribosomes in this laboratory were at variance with those of Herr and Noller (1979). The difference may be due to the fact that they had used 50S ribosomes derived from tight couples whereas the mixture of tight and loose couples was used in our experiments. Actually it has now been shown in this laboratory (Burma *et al.*, 1984b) that the two populations of 50S ribosomes are actually derived from tight and loose couples. 50S ribosomes isolated from tight couples completely lose their association capacity on treatment with kethoxal whereas those from loose couples do not lose the capacity at all on such treatment. The same is

For naked 23S RNAs or 23S RNAs isolated from treated tight and loose couple 50S ribosomes. Further, the  $Mg^{2+}$  dependent associations (with 16S RNA) of the two 23S RNAs are quite different. At  $Mg^{2+}$  concentrations below 20 mM, the association capacity of tight couple 23S RNA is more than that of loose couple 23S RNA. Further, at 2.5 mM  $Mg^{2+}$  50S ribosomes from loose couples are highly resistant to the action of RNase I whereas tight couple ones are quite susceptible. This was expected from the behaviour of kethoxal-treated 50S ribosomes. It has already been mentioned that L7/L12 stalk region of 50S ribosomes is susceptible to the action of RNase I. It is quite likely that the difference between the tight and loose couple 50S ribosomes lies in the 23S RNAs and that too in the stalk region. The 23S RNA isolated from loose couple 50S ribosomes binds more ethidium bromide than loose couple ones, as determined by fluorescence measurements (Burma *et al.*, 1984b). Further, loose couple 23S RNA is found to have slightly more hyperchromicity (on heat treatment) than tight couple 23S RNA. Thus it appears that the loose couple 50S ribosomes have more folded structure (may be in the L7/L12 stalk region) than tight couple 50S ribosomes. Not only that tight couple 50S ribosomes are more biologically active than loose couple ones, 23S RNA isolated from tight couple 50S ribosomes are also more biologically active than the loose couple 23S RNA (Burma *et al.*, 1984b) as measured by the ribosome-like activity of 16S.23S RNA complex (Burma *et al.*, 1984a). The properties of tight and loose couple 50S ribosomes as well as 23S RNAs have been summarised in table 1. It appears most likely that the major difference between tight couple and loose couple lies in the conformation of 23S RNA.

Table 1. Comparison of the properties of 50S ribosomes and 23S RNAs derived from tight couple and loose couple 70S ribosomes.

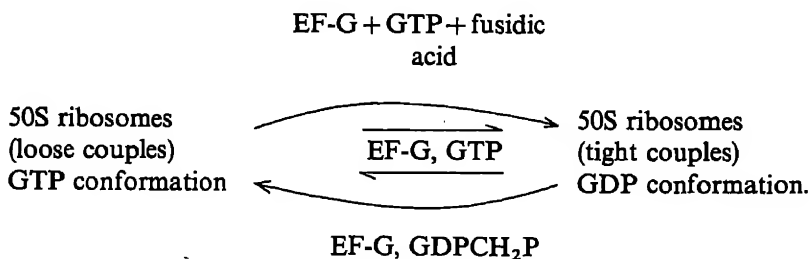
Properties	Tight couple	Loose couple
<b>50S ribosomes</b>		
Capacity to associate with 30S ribosomes at 4 mM $Mg^{2+}$	100 %	Nil
Effect of kethoxal treatment on the association capacity at 10 mM $Mg^{2+}$	Complete loss	No loss
Sensitivity to RNase I at 2.5 mM $Mg^{2+}$	Susceptible	Resistant
Biological activity	More	Less
<b>23S RNAs</b>		
Capacity to associate with 16S RNA at 4 mM $Mg^{2+}$	40 %	5 %
Effect of kethoxal treatment on the association capacity at 20 mM $Mg^{2+}$	Complete loss	No loss
Biological activity	More	Less
Binding of ethidium bromide	Less	More
Hyperchromicity on thermal denaturation	Less	More

### Interconversion of tight and loose couples

The strongest evidence in favour of the involvement of tight and loose couples in translocation came from the following unpublished results from this laboratory. On



treatment of loose couple 50S ribosomes with EF-G, GTP and fusidic acid they were converted to tight couples as tested by (i) association capacity with 30S ribosomes, (ii) effect of kethoxal treatment of this association capacity, (iii) sensitivity to RNase I, and (iv) biological activity. 23S RNA isolated from the converted 50S ribosomes also behaved like 23S RNA derived from tight couple 50S ribosomes indicating thereby that the change has taken place at the RNA level. It is well known that fusidic acid, the inhibitor of protein synthesis forms a complex with EF-G and GTP and allows the association of EF-G-GTP with 50S ribosomes. It also allows the hydrolysis of GTP but prevents the dissociation of EF-G-GDP from the ribosomes (Kuriki *et al.*, 1970; Bodley *et al.*, 1970). It should also be pointed out here that EF-G is known to bind directly to 23S RNA (Girschovich *et al.*, 1982; Skold, 1983). Therefore it is quite likely that 50S ribosome (rather 23S RNA) is locked in one conformation (tight couple one) due to treatment with EF-G, GTP and fusidic acid. The conversion of tight couples to loose couples could be effected by EF-G and non-hydrolysable GTP analogue GDPCH<sub>2</sub>P but the conversion is not so efficient. However, the treatment at 70S tight couple level instead of 50S tight couple, leads to somewhat better conversion. It is well known that the binding of GDPCH<sub>2</sub>P to 50S ribosome is rather weak although it has been claimed that translocation takes place in the absence of hydrolysis of GTP (Karizo, 1978). Thus it is expected that GDPCH<sub>2</sub>P will lock 50S ribosomes (rather 23S RNA) in the other conformation (loose couple conformation). It is also highly interesting to note that on treatment of either tight or loose couple 50S ribosomes with EF-G and GTP a mixture of the two is produced. These results clearly show that the tight and loose couple 50S ribosomes are interconvertible forms and the interconversion takes place with the help of the agents responsible for translocation. The reactions are shown below.



It is also possible to convert loose couple 23S RNA to tight couple one by heat treatment.

### Involvement of 23S RNA in translocation

Originally ribosomes were thought to be inert platform for the synthesis of the proteins but as the involvement of ribosomal proteins in the various steps of protein synthesis became gradually known it was realised that ribosomes play an active role in protein synthesis. So long ribosomal RNAs appeared to be the requisite structures for holding the ribosomal proteins in position and thus allowing the latter to function. But this

concept also needs revision as ribosomal RNAs are also found to participate directly in several reactions catalysed by ribosomes. Some of these are as follows: (i) recognition of mRNA by the 3'-end of 16S RNA (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Dunn *et al.*, 1978), (ii) binding of tRNA (Noller and Chaires, 1972; Schwarz and Ofengand, 1978; Ofengand *et al.*, 1982; Brow and Noller, 1983), (iii) antibiotic sensitivity and resistance (Helser *et al.*, 1972; Lai *et al.*, 1973; Yamada *et al.*, 1978), (iv) binding of 5S RNA by 23S RNA through proteins (Gray *et al.*, 1972; Spierer *et al.*, 1979; Tewari and Burma, 1983), (v) binding of initiation factors, at least IF3 (Gualerzi and Pon, 1973; Nag *et al.*, 1983), (vi) binding of EF-G (Girschovich *et al.*, 1982; Skold, 1983), (vii) subunit association (Nierhaus, 1982; Herr and Noller, 1978, 1979; Herr *et al.*, 1979; Shanter and Shane, 1977; Steitz, 1979; Van duin *et al.*, 1976; Nag and Burma, 1984), and (viii) binding of poly U and phenylalanyl tRNA, GTP hydrolysis, peptidyl transferase activity and polyphenylalanine synthesis as demonstrated in this laboratory (Burma *et al.*, 1984a).

Although it was suspected for quite a long time that RNA-RNA interaction plays a major role in the association of 30S and 50S subunits (references cited above) no direct evidence was, however, available. It was demonstrated for the first time in this laboratory that 16S and 23S RNAs form a specific bimolecular complex under two well-defined conditions: (i) reconstitution condition under which 30S and 50S ribosomes can be reassembled from their constituent RNAs and proteins (Traub and Nomura, 1968; Nierhaus and Dohme, 1974) and (ii) alcohol condition under which 16S and 23S RNAs assume structural features of 30S and 50S ribosomes respectively (Vasiliev *et al.*, 1978; Vasiliev and Zalite, 1980). Subsequently it was shown that 5S RNA can also be incorporated into such complex but only with the help of three ribosomal proteins, L5, L18, L15/L25 (Tewari and Burma, 1983). L5, L18 and L25 are 5S RNA binding proteins (Gray *et al.*, 1972; Horne and Erdman, 1972; Yu and Wittman, 1973) and L15 has been involved in the assembly of 5S RNA into 50S ribosomes (Nierhaus, 1982; Rohl and Nierhaus, 1982). These studies indicated the involvement of ribosomal RNAs in the association of ribosomal subunits as well as binding of 5S RNA through proteins. The former was confirmed by the kethoxal treatment of 30S and 50S ribosomes as well as 16S and 23S RNAs, the results of which have been described elsewhere (Nag and Burma, 1984). As discussed already, the kethoxal treatment led to the observation that 50S ribosomes occur in two distinct forms and that also due to two different conformations of 23S RNA. These studies further indicated the involvement of 23S RNA in the translocation reaction (Burma *et al.*, 1984b). That rRNAs have distinct roles to play in the various steps of proteins synthesis has been shown by demonstrating the ribosome-like activity of 16S.23S RNA complex (Burma *et al.*, 1984a). The various steps of proteins synthesis as well polyphenylalanine synthesis could be demonstrated with the addition of the factors involved in protein synthesis and a limited number of ribosomal proteins.

The involvement of 23S RNA in translocation was, however, indicated from an earlier observation in this laboratory (Raziuddin *et al.*, 1979). The L7/L12 stalk region of 50S ribosome was found to be susceptible to the action of single-strand specific enzyme RNase I. This was strengthened by the subsequent observation in this laboratory that 50S ribosomes become resistant to the action of RNase I on removal of L7/L12 (Byasuni and Burma, 1982), contrary to the expectation. On addition of

L7/L12 back to the core particle it again becomes susceptible to RNase I. Although it is normally believed that L7/L12 do not directly interact with 23S RNA but are attached to it through L10 (Schrier *et al.*, 1973; Tokimatsu *et al.*, 1981) it is quite logical to conclude from the above mentioned observations that L7/L12 have the capacity to organise structures of rRNA in the stalk region either by direct or indirect interaction with 23S RNA. Actually Marquardt *et al.* (1979) have shown weak interaction of L7/L12 with 23S RNA. The observations made in this laboratory are of great significance as L7/L12 are known to be directly involved in the translocation. Further, elongation factor EF-G seems to bind directly to 23S RNA at a site close to L10 (Girschovich *et al.*, 1982; Skold, 1983). Bochkareva and Girschovich (1984) have recently shown that the binding of EF-G interferes with the action of RNase I on 23S RNA near the EF-G binding region. These observations clearly indicate that 23S RNA (specially the domain present in stalk region) may be directly involved in translocation. This is further substantiated by the very recent observation in this laboratory (which has been mentioned already) that the tight couple 50S ribosomes are highly susceptible to the action of RNase I whereas loose couple 50S ribosomes are quite resistant in the presence of 2.5 mM  $Mg^{2+}$  (Burma *et al.*, 1984b). Based on the earlier observation in this laboratory (Byasuni and Burma, 1982) and the recent observation that the tight couple and loose couple 50S ribosomes are the products of translocation process itself, as evident from their interconversion by the agents responsible for translocation, it can justifiably be proposed that 23S RNA is directly involved in the translocation process. Most probably it undergoes conformational change during this process. Further, the change in conformation of 23S RNA is most likely in the stalk region and manifested in the alteration of the association capacity of 50S ribosomes with 30S ribosomes. This association takes place most probably through the interaction between 16S and 23S RNAs.

### Proposed model and justifications

Although no direct evidence can be provided at present for the mechanism of translocation, yet the observations made in this laboratory and other laboratories, as summarised above, have tempted us to provide the following tentative model for translocation (figure 2). EF-G associated with GTP (or, in other words, EF-G; GTP complex) binds directly to 23S RNA of 50S ribosome at the site close to the binding site of L10 through which proteins L7/L12 are bound to 50S ribosome. It should be pointed out here that electron microscopy of 50S particles to which EF-G was crosslinked indicated a position close to the stalk protuberance (Girschovich *et al.*, 1981). This binding induces conformational change in 23S RNA in the stalk region (the conformation induced by such binding may be designated as GTP conformation). The conformational change is most probably induced by L7/L12 which appear to be motile proteins. The motility is being assumed to be due to the interaction of EF-G-GTP with not only 23S RNA but also L10 to which L7/L12 proteins are bound. Subsequent to this event, GTP is hydrolysed to GDP and  $P_i$  through some unknown mechanism and 23S RNA goes back to original conformation (which may be called as GDP conformation). Since EF-G-GDP can not remain bound to 23S RNA it comes off and

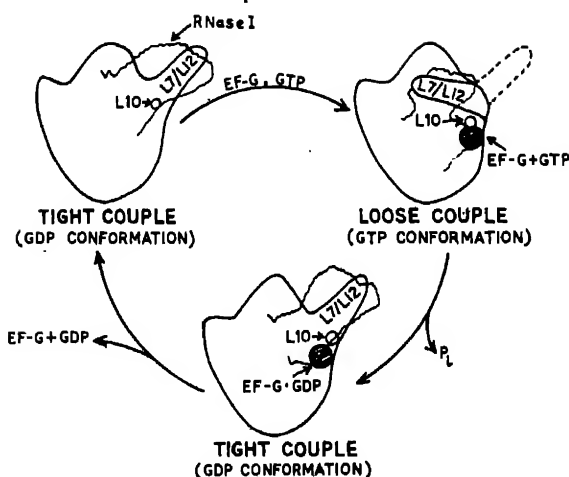


Figure 2. Model for translocation.

EF-G dissociates from GDP, reassociates with another molecule of GTP and the whole process is repeated again. The only advantage of the proposed model is that it is based on some direct experimental data and vulnerable to experimental assault.

The model is capable of explaining some of the observations made in this laboratory (table 1). Since 23S RNA in tight couple ribosomes is in unfolded form in the stalk region, it is sensitive to RNase I. Similarly, the binding of more ethidium bromide to loose couple 23S RNA and also more hyperchromicity on melting of this RNA, in comparison to tight couple 23S RNA, also fit in with the assumption of more folding of RNA in the former case.

Tight couple 50S ribosomes or 23S RNA lose their capacity to associate with 30S ribosomes and 16S RNA respectively on treatment with kethoxal due to availability of guanine residues for modification whereas in loose couple 50S ribosomes or 23S RNA such guanine residues are not available due to the folding of RNA. Further, due to this folded structure the association capacity of loose couple 50S ribosomes (or 23S RNA) is lower than that of tight couple 50S ribosomes (or 23S RNA). This is due to ready availability of the sites of association in the latter case in comparison to the former. The interconversion of loose couple and tight couple 50S ribosomes can also be explained on the basis of the proposed model. When loose couple 50S ribosomes are treated with EF-G, GTP and fusidic acid, the conformation of 23S RNA of 50S ribosomes is converted to tight couple conformation (GDP conformation) but since EF-G can not be released due to binding with the fusidic acid loose couple 23S RNA is locked in tight couple or GDP conformation. In the case of conversion of tight couple 50S ribosomes by EF-G and  $GDPCH_2P$ , the reverse situation happens, 23S RNA is locked in GTP conformation as  $GDPCH_2P$  is not hydrolysable. Similarly, on treatment of either type of 50S ribosomes with EF-G and GTP a mixture of the two are obtained due to the cyclic operation and the fact that there is equal chance of both the conformations to be produced. On heat treatment 23S RNA of loose couple 50S ribosomes are converted to

23S RNA of tight couple 50S ribosomes, most probably due to unfolding of the RNA chain in the stalk region.

The higher biological activity of tight couple 50S ribosomes (or 23S RNA) can also be explained on the basis of the proposed model. Loose couple (or GTP conformation) is a transient conformation and cannot normally occur free from EF-G and GTP but may be obtained as an artifact of the isolation procedure. EF-G and GTP are removed during isolation and as a result 23S RNA is locked in GTP conformation. EF-G and GTP normally bind to tight couple 50S ribosomes having GDP conformation which is a natural process. It is rather difficult for EF-G (and GTP) to bind to 23S RNA in the loose couple conformation due to already folded conformation of 23S RNA. Under physiological condition no such binding is expected to take place. Tight couples are thus ready for translocation whereas loose couples have to be forced to bind with EF-G and GTP for conversion to tight couple and initiation of translocation. This model also emphasises that the hydrolysis of GTP is not necessary for translocation (Kaziro, 1978). The conformational change of 23S RNA required for translocation is effected by the binding of EF-G and GTP to 50S ribosome. The hydrolysis of GTP is subsequently necessary to bring it back to the earlier conformation. Work is in progress in this laboratory to test the model still further and it is being hoped that the details of the mechanism of conformational change of 23S RNA during translocation will be learnt soon.

### Acknowledgements

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## Molecular biology of tubulin: Its interaction with drugs and genomic organization

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**Abstract.** Microtubules are ubiquitous cellular structures found in eukaryotic organisms and responsible for a variety of functions. These functions include mitosis, motility, cytoskeletal architecture, intracellular transport and secretion. The major structural component of microtubules is tubulin, a dimeric protein molecule consisting of two similar but nonidentical subunits ( $\alpha$  and  $\beta$ ) each of about molecular weight 55,000. With the introduction of radioactive colchicine for the first time it has been reported that colchicine binds specifically to tubulin. At this point microtubule research stepped up to a new era linking microtubules with other spindle poisons which are structurally diverse as well as binding at different sites on to the tubulin heterodimer. These antimicrotubular agents have already provided valuable information regarding microtubule-mediated cellular functions and its association and dissociation phenomena. Tubulins appear to be conserved proteins based on *in vitro* copolymerization and comigration on polyacrylamide gel electrophoretic properties. Further, amino acid sequences of both  $\alpha$  and  $\beta$  subunits from a variety of sources also appear to be mostly conserved. The evolutionary conservation of tubulin genes is highly reflected at the nucleic acid level as well. The estimation of the number of genes for tubulin and their organization in a variety of organisms have opened up a new dimension to microtubule and tubulin research. The multigene family for tubulins comprising also pseudogenes is suggestive that more than one gene for each  $\alpha$  and  $\beta$  tubulin is functional in the cell. Therefore, it has been speculated that different tubulin gene products contribute to functionally different microtubules at specific stages in cell cycle and cell growth. Heterogeneity in both  $\alpha$  and  $\beta$  tubulins has already been established during different stages of development of the cell. Obviously, it reflects that tubulin genes are highly regulated and this regulation might be at the transcriptional and/or translational level. Whatever is the actual control mechanism it appears that cells can detect an enhanced pool of depolymerized subunits and a rapid and specific control in tubulin gene expression at the transcriptional and/or post transcriptional level does occur.

**Keywords.** Tubulin; microtubule; drugs; tubulin mRNA; cDNA; pseudogene; evolution

### Introduction

The microtubule system has drawn attention of several workers in the recent past for its easy availability from animal brain cells, comparatively easier way of purifying tubulins to a homogeneous preparation, its easy polymerization and depolymerization *in vitro*,

Abbreviations used: SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis;  $M_r$ , molecular weight; CD, circular dichroism; CHO, chinese hamster ovary; MBC, methyl benzimidazole-2-yl carbamate; CIPC, coumarin and isopropyl-(N-3 chlorophenyl) carbamate; APM, amphoteric-methyl



its simple assay by colchicine binding reaction, preparation of cDNA and genome analysis. The main thesis of the present review is to answer (i) what is the extent of microheterogeneity in tubulins? (ii) how colchicine and its analogues bind with tubulin? (iii) what is the mechanism of substoichiometric drug poisoning of microtubule assembly? (iv) how the biosynthesis of tubulin is regulated? (v) how many functional and nonfunctional tubulin genes are present in the cell? (vi) what is the genetic complexity of tubulin genes? and (vii) what conservation of tubulin gene signifies?

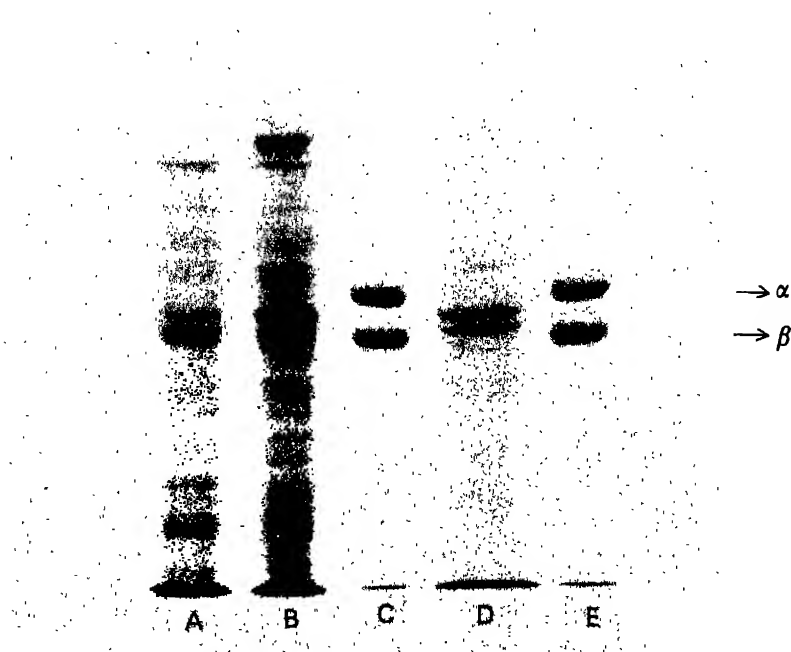
Several reviews appeared recently on different aspects of tubulin and microtubule systems (Biswas *et al.*, 1981; Carlier, 1982; Correia and Williams, 1983; Cowan and Dudley, 1983; Hill and Kirschner, 1984) but the informations regarding the questions raised above are still fragmentary and emerging. An attempt is being made here to discuss the problems enumerated and the documented information.

### **Tubulin: A ubiquitous protein**

Tubulin is the major constituent of microtubule structure found in all eukaryotic cells. Bryan and Wilson (1971) demonstrated that purified tubulin from chick embryo brain could be resolved electrophoretically into two closely located components on 8 M urea-polyacrylamide gels after reduction and acetylation. These two subunits were termed  $\alpha$  and  $\beta$  tubulins, the  $\beta$  subunit having greater electrophoretic mobility. This separation results mainly from a difference of charge, since sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the two subunits under high ionic strength and neutral pH conditions (Weber and Osborn, 1969) shows only a single band having an apparent molecular weight  $M_r$  around 55,000. However, with SDS-PAGE of lower strength and higher pH (Laemmli, 1970), separation of  $\alpha$  and  $\beta$  subunits can be achieved (Wilson and Bryan, 1974; Linck, 1976). The two subunits were found in equimolar quantities in nearly all cells studied (Dustin, 1978) and  $\beta$  tubulin was shown to have a  $M_r$  of 54,000–58,000 and  $\alpha$  tubulin, a  $M_r$  of 46,000–54,000 (Feit *et al.*, 1971; Olmsted *et al.*, 1971; Raff and Kaumeyer, 1973). These two subunits are distinct, but closely related proteins. A close relation between  $\alpha$  and  $\beta$  tubulins has been revealed by amino acid sequence analysis (Ludueno and Woodward, 1975). The 451 amino acid residues of  $\alpha$  tubulin from pig brain and 445 residues of  $\beta$  tubulin display 41 % sequence identity (Postingel *et al.*, 1983). A recent study on several taxonomically distant species (Little *et al.*, 1981) demonstrated that the  $\beta$  chain is more conserved than the  $\alpha$  chain during the course of evolution. Antisera raised against tubulin also cross react across species boundaries (Osborne and Weber, 1977). This suggests that the two polypeptides have been derived from a common ancestor protein. Each has been highly conserved in the course of evolution as indicated by the similarities of tubulins from two widely separated species, chick and sea urchin. However, antiserum against  $\beta$  tubulin does not react with  $\alpha$  tubulin (Piperano and Luck, 1977), indicating that the two subunits are distinctly different proteins. Further, they differ also in several biochemical properties. For example,  $\beta$  tubulin is phosphorylated at a serine residue (Eipper, 1972) and  $\alpha$  tubulin is tyrosinolated by tubulin tyrosine ligase (Raybin and Flavin, 1977). An enzymatic

removal, rather than the addition of tyrosin residue to  $\alpha$  tubulin has also been reported recently (Valenzuela *et al.*, 1981; Lemischka *et al.*, 1981; Cowan *et al.*, 1983). Ultrastructural data suggest that microtubules are assembled from  $\alpha\beta$  dimers. Infact, it has been ascertained that tubulin dimers are heterodimers of  $\alpha\beta$ , rather than a mixture of  $\alpha\alpha$  and  $\beta\beta$  dimers (Ludueno *et al.*, 1975).

In fact, hybrid microtubules may be formed *in vitro* by copolymerization of  $\alpha$  and  $\beta$  tubulins from different species (Snyder and McIntosh, 1976). Inspite of the similarity among tubulins from a wide variety of species, functional variation, have been observed in species to species. Thus clear immunological differences have been detected among different classes of tubulins such as outer doublets, central pair and mitotic apparatus (Mohri, 1976; Fulton *et al.*, 1971). Tubulins from different species also differ in several properties like colchicine binding (Haber *et al.*, 1972; Hart and Sabins, 1976; Davidse and Flach, 1977), *in vitro* assembly (Farrell, 1976; Langford, 1978; Murphy and Hiebsch, 1979) and immunological behaviour (Piperano and Luck, 1977; Morgan *et al.*, 1978). Recently we have isolated (manuscript in preparation) microtubule protein from higher plant *Vigna radiata* (mung bean) by streptomycin sulphate and ammonium sulphate fractionation followed by the *in vitro* polymerization of microtubule protein by Zn (II) (Banerjee *et al.*, 1982). SDS-PAGE analysis has revealed that both of the subunits of plant tubulin are different from that of brain (figure 1). Attempts to assay colchicine binding with plant tubulins using radioactive colchicine (Weisenberg *et al.*,



**Figure 1.** Purification of plant tubulin. Experimental details are as in (Sen, 1984). (A), Crude cytoplasmic supernatant; (B), ammonium sulphate fractionated protein; (C), purified goat brain tubulin; (D), purified plant tubulin; (E) same as in C.

1968) and by fluorometric method (Bhattacharyya and Wolff, 1974a) have not yet been successful in this laboratory.

### Microheterogeneity and multiplicity of tubulins

Multiple bands of either  $\alpha$  or  $\beta$  tubulin are obtained after gel electrophoresis in certain systems. This has been interpreted as due to microheterogeneity in tubulin (Bibring *et al.*, 1976; Kobayashi and Mohri, 1977). On SDS gels,  $\alpha$  occasionally splits into two bands apparently differing in charge but not in size (Bibring *et al.*, 1976; Berkowitz *et al.*, 1977; Bibring and Baxandall, 1977a,b). The splitting is observed in tubulin from sea urchin mitotic spindles and in ciliary, but not flagellar outer doublets, implying a functional significance to the putative microheterogeneity (Bibring *et al.*, 1976). Purified tubulin preparations from both Ehrlich ascites tumour cells and pig brain have been found to contain a third component migrating in between the  $\alpha$  and  $\beta$  tubulins in SDS-PAGE (Lu and Elzinga, 1977). This has been subsequently characterized as a second form of  $\beta$  tubulin, by limited proteolysis and peptide mapping (Doenges *et al.*, 1979). *Physarum* tubulin contains a subunit protein which is similar to brain  $\beta$  tubulin. However, the faster moving band on SDS gel electrophoresis in this case appearing to be altogether a different protein, after peptide mapping analysis turns out to be very similar to brain  $\alpha$  tubulin (Clayton and Gull, 1982). This raises the question on the typical nomenclature of tubulin subunits, according to electrophoretic mobility pattern *i.e.*, fast moving constitutes the  $\beta$  subunit and the slow moving one is the  $\alpha$  subunit.

There is substantial evidence, based on colchicine binding, which suggests that tubulin from lower eukaryotes may differ from that of higher organisms. In fact, the lower affinity of the tubulin from lower organisms and others for colchicine has been shown for *Tetrahymena* (Maekawa, 1978), *Aspergillus* (Davidse and Flach, 1977), *Physarum* (Roobol *et al.*, 1980) and higher plants (Sen, 1984). Brain cells display extensive tubulin microheterogeneity which has been found to be developmentally determined, increasing from seven isotubulins at birth to nine distinct components during early postnatal brain maturation (Little, 1979). These types of results can be obtained from the phosphorylation of the subunits which alter the charge on the molecule. This actually appears to be the case in axonemal tubulin from *Chlamydomonas*, where  $\alpha$  splits into five components on SDS gels and  $\beta$  into two, some of these bands differing in their degree of phosphorylation (Piperano and Luck, 1976). These arguments could also explain the splitting of  $\alpha$  on hydroxyapatite chromatography and the splitting in both  $\alpha$  and  $\beta$  on isoelectric focussing (Kobayashi and Mohri, 1977; Lu and Elzinga, 1977; Feit *et al.*, 1977a,b; Witman *et al.*, 1972). However, multiplicity of bands on electrophoresis does not necessarily imply multiplicity of amino acid sequence.

Moreover, recently genetic analysis from different laboratories reveal that for both  $\alpha$  and  $\beta$  tubulin there is more than one gene. Thus, Lopata *et al.* (1983) have reported four unique  $\beta$  tubulin genes in chicken. Mischke and Pardue (1983) showed the presence of a multigene family for  $\alpha$  in *Drosophila*. Besides these a testis specific  $\beta$  tubulin is expressed in *Drosophila* (Kemphues *et al.*, 1980, 1982; Raff and Kemphues, 1983). Thus though analysis of the data in the protein level does not signify totally the presence of

microheterogeneity, the genetic analysis reveal that there is some microheterogeneity in both  $\alpha$  and  $\beta$  tubulin. Tubulin heterogeneity in the Trypanosome, *Crithidia fasciculata* has been adequately established (Russel *et al.*, 1984). The interphase cell of *C. fasciculata* has three discrete and separable tubulin populations: the subcellular microtubule, the axonemal microtubule and the nonpolymerised cytoplasmic pool of tubulin.

### Microtubule associated proteins

Tubulin purified by assembly disassembly procedure, contains some accessory proteins which copurify with tubulin through repeated cycles of assembly and disassembly. Two classes of accessory proteins have been reported. The first one is a set of two high  $M_r$  proteins having  $M_r$  around 300,000, were termed HMW by Borisy and coworkers (Borisy *et al.*, 1975; Murphy and Borisy, 1975) and MAP by Rosenbaum and coworkers (Dentler *et al.*, 1975). Another class of low  $M_r$  accessory proteins ( $M_r$  55,000–70,000) has been reported by Kirschner and coworkers and were termed as  $\tau$  proteins (Weingarten *et al.*, 1975). The contents of accessory proteins in a preparation depends on the conditions used in the *in vitro* assembly. All these microtubule associated proteins are often collectively termed MAPs. MAPs could be separated from tubulin and are believed to be involved in the assembly of microtubules (Snyder and McIntosh, 1976). The role of MAPs in microtubule assembly has been discussed (Scheele and Borisy, 1979). Some of the enzyme activities have been observed to be associated with isolated microtubules: adenosine triphosphatase (Gelfand *et al.*, 1978), guanosine triphosphatase (David-Pfeuty *et al.*, 1977), protein kinase (Eipper, 1974), phosphoprotein phosphatase (Jameson *et al.*, 1980) nucleoside diphosphokinase (Jacobs and Huitorel, 1979), adenylate cyclase (Margolis and Wilson, 1979), 3',5'-cyclic AMP phosphodiesterase (Watanabe *et al.*, 1976), glutamate dehydrogenase (Karr *et al.*, 1979), tyrosine hydroxylase (Borisy *et al.*, 1975), alkaline phosphatase (Prus and Wallin, 1983) and DNA polymerase (Avila, 1980).

### Interaction of tubulins with drugs

Several antitumour drugs, *viz.*, colchicine and its analogues podophyllotoxin and vinca alkaloids inhibit mitosis and other cellular functions by specifically binding to tubulin and inhibiting its assembly into microtubule. Consequently, these drugs have become valuable tools in understanding the role of microtubules in diverse cellular functions.

#### *Colchicine and its structural analogues*

Colchicine is one of the oldest drugs in the pharmacopoeia and has been named after the meadow saffron *Colchicum autumnale* (Eigsti and Dustin, 1955). It is a tropolone derivative with three rings—one trimethoxybenzene ring (A-ring), one saturated seven membered carbon ring (B-ring) with a substituted acetamido group and a tropolone ring (C-ring) with one oxo and one methoxy group (figure 2) (Margulis, 1975).

The binding of colchicine with tubulin is one of the primary characteristics of the

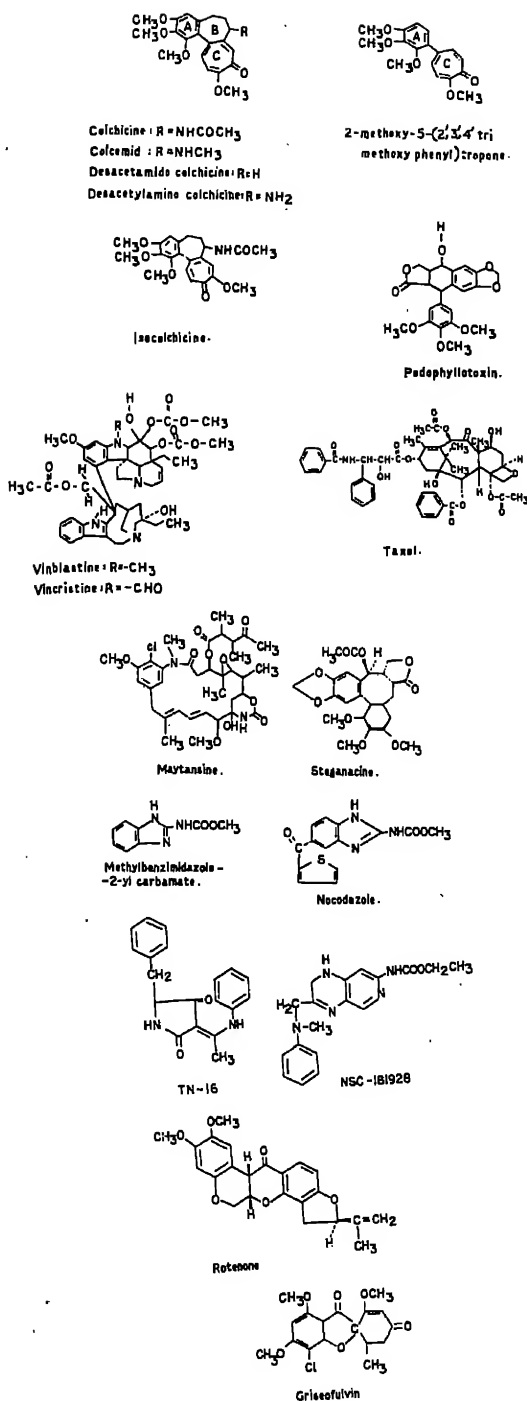


Figure 2. Chemical structures of the tubulin binding drugs.

protein and colchicine binding has been studied with tubulin prepared from many organisms and the higher plants (Flanagan and Warr, 1977; Hart and Sabins, 1973; Luduena *et al.*, 1976; Miller, 1973; Stephens, 1977; Wilson and Friedkin, 1967; Wilson and Meza, 1973). Since, the binding reaction is highly specific and the colchicine-tubulin complex is very stable, [ $^3\text{H}$ ]-colchicine can be used as an assay for quantitative analysis of the binding reaction. To measure colchicine binding, various methods have been used which include gel filtration, filtration through DEAE cellulose filters, adsorption by charcoal (Borisov, 1972; Rappaport *et al.*, 1975; Wilson and Bryan, 1974). Bhattacharyya and Wolff (1974a) have developed another unique method based on the promotion of fluorescence upon binding of colchicine to tubulin. This method does not require the separation of the free ligand, since unbound colchicine does not have any fluorescence. This method thus permits the measurement of kinetic and thermodynamic parameters under equilibrium conditions.

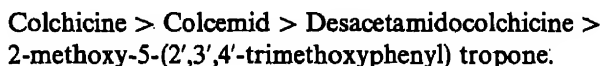
The tubulin dimer has one binding site for colchicine (Weisenberg *et al.*, 1968; Shelansky and Taylor, 1967; Wilson *et al.*, 1974). The dissociation constants,  $K_d$ , in animal tubulins are in the range of  $3.0\text{--}9.1 \times 10^{-7}$  M when measured by equilibrium method (Barnes *et al.*, 1977; Borisov and Taylor, 1967b; McClure and Paulson, 1977; Owellen *et al.*, 1974; Sherline *et al.*, 1975); when measured by kinetic methods, however, the dissociation constant is an order of magnitude lower (McClure and Paulson, 1977; Sherline *et al.*, 1975; Bhattacharyya and Wolff, 1976b; Garland and Teller, 1975). The interaction between colchicine and tubulin is noncovalent and the drug is not altered chemically upon binding (Wilson and Friedkin, 1967; Borisov and Taylor, 1967a). The binding reaction is slow (Wilson and Bryan, 1974; Bhattacharyya and Wolff, 1974b) and almost irreversible in nature. Garland (1978) proposed a two step mechanism for the interaction of colchicine with tubulin: after a fast pre-equilibration step, a slow conformational change in the tubulin molecule leads to the formation of the fluorescent complex. Engelborghs's group (Lambeir and Engelborghs, 1981) by using a fluorescence stopped flow technique, arrived at the same conclusion as made by Garland. Ventilla *et al.*, (1972) on the basis of the circular dichroic studies, have also demonstrated that colchicine binding leads to a conformational change of tubulin molecule.

The colchicine binding site on tubulin is subject to decay whose properties have been intensively studied. The decay is an all or none phenomenon; the binding site simply disappears with first order kinetics, without a gradual change in affinity. The half life of decay of mammalian tubulin is about 5–7 h (McClure and Paulson, 1977; Sherline *et al.*, 1975; Solomon *et al.*, 1973), but solubilized sea urchin outer doublet tubulin decays with a half time of 5.2–5.6 h (Wilson and Meza, 1973). This decay is stabilized by salt, vinblastine, GTP, glycerol, sucrose, and dithiothreitol and by colchicine itself (Wilson and Meza, 1973; McClure and Paulson, 1977; Sherline *et al.*, 1975; Solomon *et al.*, 1973). The decay rate is linearly influenced by temperature and pH, being most stable at  $0^\circ\text{C}$  and pH 6.75 (Wilson, 1970).

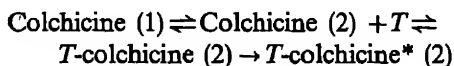
Fluorescence studies using various analogues and derivatives of colchicine have revealed that at least two moieties on the colchicine molecule are involved in the binding to tubulin (Bhattacharyya and Wolff, 1974a). One is the A-ring in which insertion of a bulky group causes complete loss of binding. The other moiety is the tropolone ring or C-ring (figure 2). It has been suggested that C-ring in combination with A-ring might be

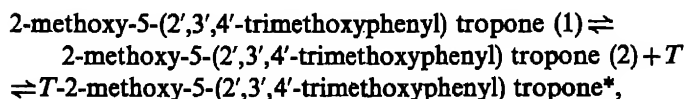
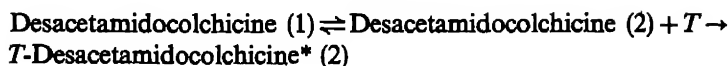
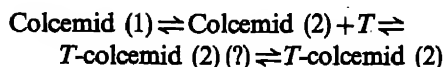
responsible for the promotion of fluorescence. Replacement of tropolone ring by a phenyl ring in colchicine causes complete loss of binding (Zweig and Chignell, 1973). Correct positioning of the carbonyl moiety in C-ring appears to be necessary also. Modification in the C-ring (as in lumicolchicine) also leads to the complete loss of binding (Wilson, 1970). On the other hand, colchicine analogues modified at the B-ring moiety are known to have potent antimitotic activity which apparently rules out any major role of this moiety in the binding of colchicine to tubulin (Fitzgerald, 1976). But it has been shown from this laboratory that a minor change in the B-ring substituent may significantly affect the mechanism as well as the nature of binding. Thus, colcemid binds to tubulin fairly rapidly and reversibly, unlike colchicine (Banerjee and Bhattacharyya, 1979). Recently, we have shown (Ray *et al.*, 1984) that tubulin has two distinct colcemid binding sites. One site has a very high affinity while the other has low affinity. The affinity constants are respectively  $1.3 \times 10^{-5} \text{ M}^{-1}$  and  $0.7 \times 10^{-5} \text{ M}^{-1}$ . The activation energy of the colcemid binding to tubulin has been found to be  $9.8 \text{ K}_{\text{cal}}/\text{mol}$ , a value lower than that for colchicine ( $19.5 \text{ K}_{\text{cal}}/\text{mol}$ ) (Ghosh Choudhury, 1984).

Recently, from circular dichroism (CD) studies, Detrich *et al.* (1981) have shown that when colchicine binds to tubulin, the 340 nm CD band vanishes, from which they concluded that a conformational change in the colchicine molecule is taking place. Moreover, the binding of colchicine to tubulin induces a change from conformation 1 to conformation 2 which provides a potential explanation for the enhancement of colchicine fluorescence (Bhattacharyya and Wolff, 1974a; Detrich *et al.*, 1981). However, it has been recently reported from this laboratory (Ghosh Choudhury *et al.*, 1983a,b) that the two colchicine analogues desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (figure 2) can bind to tubulin with a stoichiometry of one. The affinity constants for both of these drugs are  $1.6 \times 10^6 \text{ M}^{-1}$  and  $0.58 \times 10^6 \text{ M}^{-1}$  respectively. The activation energies for binding of these drugs to tubulin have been determined (Ghosh Choudhury, 1984). The values are  $6.4 \text{ K}_{\text{cal}}/\text{mol}$  for both desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone respectively. From Dreiding model building and energy calculation it has been found in this laboratory (Ghosh Choudhury, 1984) that for binding and promotion of fluorescence, both of these analogues also require a conformational change like that of colchicine. Thus the activation energies for binding of these drugs to tubulin decrease in the order:



Moreover, 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone can bind tubulin even at  $4^\circ\text{C}$  (Ray *et al.*, 1981; Ghosh Choudhury, 1984). This indicates that atleast for this drug, tubulin does not require the so called time and temperature dependent conformational change (Lambeir and Engelborghs, 1981) like that needed in the case of colchicine. In the case of desacetamidocolchicine the case is nearly similar to that of 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (activation energy =  $6.4 \text{ K}_{\text{cal}}/\text{mol}$ ). So from the existing literature and from a systematic and rigorous study in our laboratory the colchicine binding reaction may be postulated as:





where 1 and 2 are the conformation 1 and conformation 2 of the drugs and asterics represents the fluorescent species of drug-tubulin complex.

On the basis of the assumption that colchicine behaves as a bifunctional ligand during binding to tubulin, Andrew and Timasheff (1982) postulated a model in which they suggested that ring C of colchicine (figure 2) binds first and this binding would induce in the protein a conformational change bringing the A-ring domain to proper position to bind the ring. But the discrepancy in this model is that there is no room for B-ring or B-ring substituent (acetamido group). But recent studies from this laboratory (Ray *et al.*, 1981; Ghosh Choudhury *et al.*, 1983a,b; Ghosh Choudhury, 1984) have revealed that there is a vast difference in activation energy and rate of binding among colchicine and its B-ring analogues. This finding leads to a conclusion that not only A and C-ring, but B-ring of colchicine also plays a major role in its binding to tubulin and determines some of the characteristics of the tubulin-colchicine interaction.

However, it has not been established which of the subunits of tubulin carries the colchicine binding site. The report by Roussett and Wolff (1980a) had indicated that the dimeric state of tubulin was not necessary for colchicine binding and that the colchicine binding site is located on either the  $\alpha$  or  $\beta$  subunit. It has been found that lactoperoxidase, an enzyme used in the iodination of proteins, binds tubulin and dissociates  $\alpha$  and  $\beta$  monomers as  $\alpha$ -tubulin-lactoperoxidase and  $\beta$ -tubulin-lactoperoxidase complexes (Rousset and Wolff, 1980b). They found that the colchicine binding activity of tubulin was virtually unaltered after complete dissociation of subunits. It is known that the yeast tubulin can not bind colchicine (Haber *et al.*, 1972) and yeast tubulin has been reported to contain an altered  $\beta$  subunit (Clayton *et al.*, 1979). In this context it is note worthy that  $\alpha$  and  $\beta$  tubulins from a protease deficient strain of *Saccharomyces cerevisiae* comigrate with brain  $\alpha$  and  $\beta$  tubulins (Ghosh Choudhury and B. B. Biswas, unpublished observation). Colchicine resistant mutants from chinese hamster ovary (CHO) cells have been found to possess an altered  $\beta$  subunit (Cabral *et al.*, 1980). All these information collectively indicate the presence of the colchicine binding site on the  $\beta$  subunit.

Colchicine binds only to tubulin dimers but not to intact microtubules. It has been demonstrated that the colchicine binding site of tubulin gets burried within the microtubule (Sherline *et al.*, 1975; Wilson and Meza, 1973; Wilson *et al.*, 1974). The anti-microtubular action of colchicine, therefore, believed to be mediated through an inhibition of microtubule assembly rather than a direct interaction with the microtubules (Wilson, 1975; Margolis and Wilson, 1977) which exist in a dynamic equilibrium with the subunit protein tubulin (Inoue and Sato, 1967). Thus, in the presence of colchicine, soluble tubulin gets complexed with the drug and becomes



inactive for microtubule formation, which in turn shifts the equilibrium and leads to the disassembly of microtubules.

### *Podophyllotoxin*

The antimitotic drug podophyllotoxin (figure 2) is extracted from the root of the May apple *Podophyllum peltatum* (Kelly and Hartwell, 1954). In general the antimitotic activity of podophyllotoxin is qualitatively indistinguishable from that of colchicine (Pfeffer *et al.*, 1976; Wilson *et al.*, 1976; Cortese *et al.*, 1977). Although colchicine and podophyllotoxin share the same binding site on tubulin perhaps because of common trimethoxy benzene moiety (A-ring; figure 2) the mechanism of binding of podophyllotoxin appears to be somewhat different from that of colchicine as shown by Cortese *et al.* (1977). Thus, (i) podophyllotoxin binds very rapidly, about 10 times as fast as colchicine; (ii) podophyllotoxin binds readily at 0°C; (iii) podophyllotoxin binding is freely reversible (Wilson *et al.*, 1974; Wilson, 1975; Cortese *et al.*, 1977). The association rate constant for podophyllotoxin binding to rat brain tubulin has been found to be  $3.8 \times 10^6 \text{ M}^{-1} \text{ h}^{-1}$ . The dissociation rate constant is  $1.9 \text{ h}^{-1}$ .

### *Vinca alkaloids*

Vinblastine and vincristine (figure 2) are potent antimitotic drugs obtained from the plant (*Catharanthus roseus*) G. Don (*Vinca rosea* L.) and are used in the treatment of neoplastic diseases. Vinblastine binds to tubulin at sites distinct from colchicine and podophyllotoxin (Bryan, 1972). The vinblastine binding reaction is very different from that of colchicine; the binding is reversible, temperature independent and rapid (Wilson, 1975; Mandelbaum-Shavit *et al.*, 1976; Owellen *et al.*, 1972; Wilson *et al.*, 1975, 1978). The binding reaction is not affected by GTP, colchicine or calcium, although colchicine and sucrose stabilise the decay of the site (Owells *et al.*, 1972; Wilson *et al.*, 1975, 1978; Bhattacharyya and Wolff, 1976a). The decay is first order with a half time of 3.5 h and is unusual in that it affects the affinity as well as the number of sites available (Wilson *et al.*, 1978; Bhattacharyya and Wolff, 1976a). Two high affinity vinblastine binding sites with about the same affinity have been reported (Lee *et al.*, 1975; Wilson *et al.*, 1975). In contrast Bhattacharyya and Wolff (1976a) found two binding sites, differing in affinity. The reported vinblastine dissociation constants for mammalian tubulin range from  $1.25 \times 10^{-7} \text{ M}$  to  $4.5 \times 10^{-5} \text{ M}$  (Owells *et al.*, 1972, 1974; Lee *et al.*, 1975; Bhattacharyya and Wolff, 1975).

In addition to high affinity sites, there may be several low affinity binding sites for vinblastine, about 20–30 for chick brain tubulin (Wilson, 1975). Vinblastine induces tubulin to aggregate into a series of highly organized structures. Vinblastine ( $2 \times 10^{-5} \text{ M}$ ) causes tubulin to dimerize (Lee *et al.*, 1975); at  $1.0 \times 10^{-4} \text{ M}$  vinblastine, larger structures are seen, including ring, spirals and double helical structure called a macro-tubule, consisting of two spirals with a centre-to-centre spacing of 18–28 nm (Erickson, 1975; Fujiwara and Tilney, 1975; Maratz and Shelansky, 1970). In some preparations, closely packed arrays of macro-tubules constitute a crystal, which can also be induced in some cells *in vivo* (Maratz and Shelansky, 1970; Bensch and Malawista, 1969; Bensch *et al.*, 1969; Bryan, 1971).

### Griseofulvin

Griseofulvin (figure 2) is a widely used antifungal antibiotic isolated from *Penicillium griseofulvum*. It arrests mitosis and causes disorientation of microtubules *in vivo* and can prevent microtubule assembly *in vitro* (Grisham *et al.*, 1973a; Gull and Trinci, 1974). The  $K_i$  of griseofulvin for preventing assembly of recycled bovine brain microtubules is  $5.0\text{--}6.7 \times 10^{-6}$  M (Wilson *et al.*, 1975). There are contradictions about the specific binding of this drug to tubulin. It has been reported that [ $^3\text{H}$ ]-griseofulvin does not bind to pure tubulin (Grisham *et al.*, 1973b). On the other hand Roobol *et al.* (1977) have shown that griseofulvin binds to a fraction containing MAPs.

### Taxol

Recently, it has been suggested that the antimitotic drug taxol (figure 2) an alkaloid from the plant *Taxus brevifolia* acts by a somewhat different mechanism. In contrast to other drugs mentioned, taxol acts as a promoter of microtubule assembly *in vitro* and renders microtubules resistant to depolymerization by cold ( $4^\circ\text{C}$ ) and  $\text{Ca}^{2+}$  *in vitro* and tissue culture cells (Schiff *et al.*, 1979; Schiff and Horwitz, 1980). Taxol specifically inhibits cell separation in *Trypanosoma cruzi*. However, this taxol treatment permits continued multiplication of cellular organelles, including the nucleus, kinetoplast and flagellum. This observation suggests the presence of at least two classes of microtubules in *Trypanosoma cruzi* as determined by taxol sensitivity (Baum *et al.*, 1981). Tritium labelled taxol binds directly to microtubules *in vitro* with a stoichiometry approaching one (Parness and Horwitz, 1981). [ $^3\text{H}$ ]-Taxol binds to the macrophage like cell line, J 774-2 in a specific and saturable manner. Scatchard analysis of the species binding data demonstrates a single set of higher affinity binding sites (Manfredi *et al.*, 1982). Using ultra-violet mutagen treated CHO cells, Cabral *et al.* (1981) selected taxol resistant cells. By two dimensional gel electrophoresis and peptide mapping these authors reported that the taxol resistant cells contain an altered  $\alpha$  tubulin. This indicates that the taxol binding site may be on the  $\alpha$  tubulin. From a study in this laboratory, some of the characteristics of the taxol induced purified tubulin polymerization *in vitro* have been elucidated (Ghosh Choudhury *et al.*, 1983b; Ghosh Choudhury, 1984). The antimitotic drug colchicine inhibits taxol induced purified tubulin polymerization *in vitro* with a  $K_i$  of  $1.5 \times 10^{-6}$  M. Moreover, like normal microtubules, colchicine binding site is buried in taxol induced polymers. Unlike normal assembly, however, taxol induced assembly is not inhibited by the colchicine analogues like desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone. Rather this analogue tubulin complex can copolymerize in the presence of taxol into microtubules. Moreover, from a rigorous thermodynamic analysis it has been shown that like normal MAPs induced microtubule assembly, taxol induced purified tubulin polymerization is also an entropically driven process (Johnson and Borisy, 1979; Ghosh Choudhury *et al.*, 1983b; Ghosh Choudhury, 1984).

### Other antimicrotubular drugs

Benzimidazole derivatives which were first introduced as fungicides, are another group of important microtubule poisons. These include methyl benzimidazole-2-yl carbamate

(MBC) (figure 2), mebendazole, and oncodazole or nocodazole (Davidse and Flach, 1977). These drugs exhibit antimitotic action similar to colchicine. A unique feature of MBC is that it selectively destroys the microtubules of parasitic worms (Borgers *et al.*, 1975) without any effect on those of the host. Moreover, it does not affect the *in vitro* assembly of porcine brain tubulin (Davidse and Flach, 1978). Nocodazole shows potent antimitotic activity in mammalian system and also inhibits the *in vitro* microtubule assembly (DeBrabander *et al.*, 1976). It competes with colchicine for the binding site on tubulin. It is noteworthy to mention that nocodazole has got similarity with colchicine only with its *B*-ring moiety and both of them induce a change in the conformation of tubulin molecule after binding (Lee *et al.*, 1980).

Another important antimicrotubular agent is steganacin (figure 2) which has been extracted from the wood and stems of *Steganotaenia araliaces* Hochst. It blocks *Hela* cell replication in mitosis. This drug is a competitive inhibitor of colchicine binding to tubulin and inhibits microtubule assembly *in vitro* (Schiff *et al.*, 1978; Schiff and Horwitz, 1981).

A potent antileukemic macrolide maytansine (figure 2), isolated from *Maytenus ovatus*, inhibits mitosis and also the *in vitro* assembly of microtubules (Remillard *et al.*, 1975). It competes with vinblastine and vincristine for their high affinity binding site on tubulin but does not lead to the formation of tubulin crystals (Bhattacharyya and Wolff, 1977).

Rotenone (figure 2) is another potent antimitotic drug which interacts reversibly with the colchicine binding site on tubulin and inhibits *in vitro* assembly of microtubules (Brinkley *et al.*, 1974; Marshall and Himes, 1978). Besides these, recently two compounds TN-16 (Arai, 1983) and antileprosy drug dopasone (Rajagopalan and Gurnani, 1983) have been reported to bind tubulin and inhibit microtubule assembly. Coumarin and isopropyl-(*N*-3 chlorophenyl) carbamate (CIPC) have been found to affect the mitotic division in lower eukaryotes suggesting that these have antimitotic activity (Katz *et al.*, 1982). Coumarin has effects similar to those produced by antimicrotubule agents such as colchicine, CIPC and benzimidazole derivatives (Welker, 1982). Coumarin apparently does not affect spindle microtubules. This different specificity for cytoplasmic and spindle microtubules might be used to probe the microtubule functions and its heterogeneity.

In the case of plants the herbicides, amiprofos-methyl (APM) have been found to poison specifically microtubule dynamics (Morejohn and Fosket, 1984). The potent antimicrotubule action of APM has already been used to investigate the regulation of tubulin synthesis in *Chlamydomonas* (Collis and Weeks, 1978). Characteristic features of some important antimitotic drugs have been presented in table 1.

### Mechanism of substoichiometric drug poisoning of microtubule assembly

Antimicrotubular drugs like colchicine, vinblastine and podophyllotoxin inhibit microtubule assembly both *in vivo* and *in vitro* in a substoichiometric manner, *i.e.* concentrations of drugs needed to inhibit the assembly is far substoichiometric to the free tubulin concentration in solution (Olmsted and Borisy, 1973; Wilson *et al.*, 1976). The drug-tubulin complex rather than the drug itself, has been found to be the

Table 1. Some kinetic and thermodynamic parameters of drug tubulin interaction.

Drug	No. of binding sites	Affinity constant	Activation energy	References
Colchicine <sup>a</sup>	1	$3.33-1.09 \times 10^6 \text{ M}^{-1}$	$19.5 \text{ K}_{\text{cal}}/\text{mol}$	Barnes <i>et al.</i> (1977), Borisy and Taylor (1967b), McClure and Paulson (1977), Owellen <i>et al.</i> (1974), Sherline <i>et al.</i> (1975), Ghosh Choudhury (1984).
Colcemid <sup>a</sup>	2	$0.7 \times 10^5 \text{ M}^{-1}$ $1.3 \times 10^5 \text{ M}^{-1}$	$9.8 \text{ K}_{\text{cal}}/\text{mol}$	Ray (1980), Ghosh Choudhury (1984), Ray <i>et al.</i> (1984).
Desacetamido	1	$1.6 \times 10^6 \text{ M}^{-1}$	$6.4 \text{ K}_{\text{cal}}/\text{mol}$	Ray <i>et al.</i> (1980), Ghosh Choudhury <i>et al.</i> (1983a), Ghosh Choudhury (1984).
2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone <sup>a</sup>	1	$0.58 \times 10^6 \text{ M}^{-1}$	$4 \text{ K}_{\text{cal}}/\text{mol}$	Ray <i>et al.</i> (1980), Ghosh Choudhury <i>et al.</i> (1983a), Ghosh Choudhury (1984).
Podophyllotoxin <sup>a</sup>	1	$1.8 \times 10^6 \text{ M}^{-1}$	$14.7 \text{ K}_{\text{cal}}/\text{mol}$	Cortese <i>et al.</i> (1977).
Vinblastin <sup>b</sup>	2 and/or more	$6.2 \times 10^6 \text{ M}^{-1}$ $8 \times 10^4 \text{ M}^{-1}$	N.D.	Bhattacharyya and Wolff (1976a).
Vincristine <sup>b</sup>	1	$1.8 \times 10^7 \text{ M}^{-1}$	N.D.	York <i>et al.</i> (1981)
Maytansine <sup>b</sup>	1	N.D.	N.D.	Bhattacharyya and Wolff (1977)
Steganacin <sup>a</sup>	1	N.D.	N.D.	Schiff <i>et al.</i> (1978), Schiff and Horwitz (1981)
Nocodazole <sup>a</sup>	1	N.D.	N.D.	Lee <i>et al.</i> (1980)
Methyl-benzimidazole-2-yl carbamate <sup>a</sup>	1	N.D.	N.D.	Borgers <i>et al.</i> (1975), Davidse and Flach (1977).
NSC-181928 <sup>a</sup>	1	N.D.	N.D.	Hamel and Lin (1982).
Rotenone <sup>a</sup>	1	N.D.	N.D.	Brinkley <i>et al.</i> (1974), Marshall and Himes (1978).
TN-16 <sup>a</sup>	1	N.D.	N.D.	Arai (1983).
Taxol	1	N.D.	N.D.	Parness and Horwitz (1981).
Dopasone	N.D.	N.D.	N.D.	Rajagopalan and Gurnani (1983).

<sup>a,b</sup> Indicate competitive binding site for colchicine and vinblastine respectively. N.D., Not determined.

inhibitory species (Margolis and Wilson, 1977). *In vitro* assembly is inhibited half maximally when only 2% of the unpolymerized tubulin is complexed with the drug.

The mechanism by which colchicine exhibits substoichiometric poisoning, has been studied by several workers in recent years. Margolis and Wilson (1977) demonstrated that colchicine-tubulin dimer (CD) complex adds on to the growing end of the

microtubule and irreversibly caps that end so that further addition of dimers becomes impossible. Microtubule assembly would therefore be poisoned owing to a reduction in the number of the assembly competent ends. Alternatively, Sternlicht and Ringel (1979) demonstrated that CD complex decreases the affinity of a microtubule end for further dimer addition. In this case, the poisoning of assembly results owing to a decrease in the apparent rate of dimer addition of microtubules without any decrease in the number of the assembly competent ends.

Lambeir and Engelborghs (1980) have reported that the binding of CD to microtubule ends is reversible and the affinity of tubulin and CD for microtubule ends is of the same order of magnitude. Farrell and Wilson (1980) have re-examined the poisoning mechanism and found that the binding of CD complex to microtubule end was not irreversible. In time, some free tubulin dimer addition occurs over the CD block, ultimately resulting in recovery from that block. The recovery depends on the molar frequency of the addition of free tubulin dimers to that of CD complex. At high tubulin/CD ratios ( $> 250$ ) the recovery is essentially complete and copolymer formation occurs without a reduction in the number of assembly competent ends. However, at a very low ratios ( $< 14$ ), frequency of addition of free tubulin dimers to that of CD complex falls below a critical ratio, and under such condition the inhibition of assembly becomes almost complete and the number of assembly competent end is reduced to zero.

Deery and Weisenberg (1981) have suggested that it is the colchicine-tubulin oligomer complex that is the actual inhibitory species. According to their model, the elongation of microtubules requires the addition of tubulin oligomers to the end of the microtubule. The presence of a single colchicine molecule to the end subunit of a linear oligomer could lead to an inhibition of the interaction of oligomers with the microtubule end. The extent of inhibition may depend upon the fraction of tubulin subunits in an oligomer that contain colchicine. Colchicine containing subunits might also inhibit the lateral co-operative interactions between the subunits of adjacent oligomers.

### **Tubulin mRNA during growth and development**

It has been observed that tubulin synthesis can be regulated in the presence of drugs. Drugs (colchicine and nocodazole) that depolymerize microtubule decreases and those (taxol and vinblastine) favouring polymerization increase the synthesis of tubulin mRNA (Cleveland *et al.*, 1981).

Initial experiments have shown that mRNA fractions separated from the total mRNA of chick embryo brain by sucrose density gradient centrifugation could be translated *in vitro* in rabbit reticulocyte cell-free lysates containing [ $^{35}\text{S}$ ]-methionine. Both actin and tubulin were detected in the product by electrophoresis (Gilmore-Herbert and Heywood, 1976). Recently, Kirschner and his associates have described the isolation of two mRNAs coding for  $\alpha$  and  $\beta$  tubulins by fractionation of poly (A) containing RNA from embryonic chick brain, thus giving evidence for two tubulin genes (Cleveland *et al.*, 1978). These two mRNAs are very similar in  $M_r$  (650,000) as judged by mobility on denaturing gels containing methylmercury. However, as

reported by Bryan *et al.* (1978), the separate mRNAs coding for the  $\alpha$  and  $\beta$  subunits were resolved on native gels,  $\alpha$  migrating faster than  $\beta$ , indicating that  $\alpha$  mRNA had more secondary structure than that of  $\beta$  mRNA. It has also been shown by Cleveland *et al.* (1978) that tubulin is synthesized in both free and membrane-associated polysomes. Peptide mapping in all cases confirmed that the *in vitro* translation products are  $\alpha$  and  $\beta$  tubulins, which can coassemble into microtubules with added carrier microtubule proteins. Similarly, evidence for the synthesis of tubulin on membrane bound and free ribosomes from rat brain has been shown. In our laboratory it has been observed that colchicine can bind with the polysomes isolated from rat brain. Tubulin messenger RNA thus enriched from polysomes was used for cDNA synthesis and cloning (Chakraborty *et al.*, 1983; Sen *et al.*, 1984). Tubulin has also been detected among the *in vitro* translation products of poly (A) containing RNA from *Tetrahymena* in cell free wheat germ and rabbit reticulocyte systems. Isoelectric focusing of the products reveals heterogeneity in both  $\alpha$  and  $\beta$  tubulins (Portier *et al.*, 1979). Similar heterogeneity has also been observed in rat brain tubulins synthesized *in vivo* or by *in vitro* translation of rat brain RNA in a wheat germ or rabbit reticulocyte cell-free system (Saborio *et al.*, 1978).

Gozes *et al.* (1980) have reported that prenatal rat brain tubulin can be resolved by isoelectric focusing into five or six components, while in mature brain nine distinct forms of tubulin were evident. Also, tubulins isolated from various regions of the brain displayed a quantitative difference in their microheterogeneity. Obviously, the question arises whether such microheterogeneity results from post-translational modification or from multiple species of mRNA. Gozes *et al.* (1980) have shown that mature brain mRNA translated *in vitro* results in the synthesis of five tubulin forms. The mRNA could be resolved into several species coding for these distinct tubulin forms. An age dependent enhancement in the relative translation of the mRNA coding for a particular tubulin species has also been observed, this being characteristic of mature brain and not apparent among the *in vitro* translation products of prenatal mRNA. These results clearly indicate that at least some of the variations in tubulin microheterogeneity may be controlled at the mRNA level.

Although the flagellar tubulin of *Naegleria* has been shown to be similar to tubulins isolated from other sources, it is interesting to note that antibodies prepared against the outer doublet tubulin react only with the flagellar tubulin and not even with the tubulin of the amoeboid form (Kowit and Fulton, 1974). Using this specific antibody, Lai *et al.* (1979) have demonstrated that during differentiation of an amoebae to a flagellate, the flagellar tubulin was the predominant product of translation in a wheat germ cell free system as directed by the poly (A) containing RNA extracted from these differentiating cells. However, no flagellar tubulin or tubulin message could be detected by the translation assay or using  $\beta$  tubulin clone probe (Fulton and Lai, 1982) against RNA extracted from amoebae prior to differentiation. These facts indicate that flagellar tubulin mRNA appears only during differentiation. However, it is not settled whether such appearance of translatable mRNA arises from *de novo* synthesis of mRNA or from post transcriptional processing of a preexisting mRNA species to a translatable form.

A similar marked increase in the amount of translatable tubulin mRNA has also been observed during the regeneration of flagella by gametes of *Chlamydomonas reinhardtii*

(Weeks and Collis, 1976). Of course, a major difference between the *Chlamydomonas* and *Naegleria* systems is that while the gametes of the former species contain preexisting flagella and sufficient tubulin for about 50% flagellation by length in the absence of protein synthesis (Weeks and Collis, 1976; Lafebvre *et al.*, 1978), in the latter system no flagella preexist in the amoebae nor does any flagellation occur in the absence of prior protein synthesis. However, in both systems, flagellation during regeneration or differentiation is accompanied by an increase in translatable tubulin mRNA within 15–20 min after the stimulus, reaching a maximum amount at about 1 h and then declining as regeneration or differentiation is completed (Lai *et al.*, 1979). Using cDNA probe for hybridization with RNA, it was observed that tubulin sequences in RNA increased within 8 min following deflagellation of *Chlamydomonas reinhardtii*, reached maximal levels by 50 min and then began to decrease by 80 min after deflagellation. One hybridization band was detected with use of the  $\beta$  tubulin probe, but two RNA size classes hybridised to the  $\alpha$  tubulin probe (Silflow and Rosenbaum, 1981). From their study, it appears that tubulin synthesis after deflagellation is regulated essentially at transcriptional level.

Gene expression and mRNA of tubulin have been studied in ciliated protozoa *Tetrahymena pyriformis* (Fliss and Suyama, 1979; Zimmerman *et al.*, 1983). The electrophoretic mobility of  $\alpha$  tubulin subunit on SDS-PAGE differs but  $\beta$  tubulin subunits have the same mobility. *In vitro* synthesized tubulin was found to have exactly the same mobility as well. The pattern of fluctuation of tubulin mRNA was studied by *in vitro* translation and by hybridization with tubulin probe during cell cycle. This suggests that as the cell progresses through the cell cycle, tubulin synthesis is controlled at the mRNA level.

Tubulin gene expression has been extensively studied in *Drosophila melanogaster*. During embryonic development, in *Drosophila*  $\beta_3$  tubulin subunit ( $\beta$  tubulin variant) is expressed transiently with concomitant increase of  $\alpha$  tubulin in order to keep  $\alpha$  and  $\beta$  tubulin ratio constant (Raff *et al.*, 1982). A testis specific  $\beta$  tubulin has also been identified which is used in constructing the motile sperm tail (Kemphues *et al.*, 1979). However, the products of this testis specific gene are not restricted to a single functional class. A family of four  $\alpha$  tubulin genes have been isolated having difference in nucleotide sequence and atleast three of them have different patterns of transcription during *Drosophila* development. In addition, atleast two of the genes have more than one RNA product. The concentrations of the different RNA products from a single gene also vary independently during the development of the organism (Kalfayan and Wensink, 1981).

Hybridization of cloned cDNA probes of sea urchin, *Laytherhimus pictus* to filter blots of RNA from developmental stages show that tubulin synthesis is regulated at translational level as well as by the variations in the accumulation of different tubulin transcripts. The  $\alpha$  sequences in the egg reside primarily in RNAs that are larger (2.8 and 2.5 kb) than the mature forms of the mRNA (1.75–2.2 kb); these are converted to the smaller forms after fertilization. In contrast,  $\beta$  mRNAs (1.8–2.2 kb) do not undergo any obvious size reduction after fertilization (Alexandraki and Ruderman, 1981). Some authors demonstrated the existence of atleast three different  $\beta$  tubulin mRNA and also developmental regulation of different  $\alpha$  and  $\beta$  tubulin sequences.

Autoregulatory mechanism of control of expression of  $\alpha$  and  $\beta$  tubulin has been shown

by Ben Ze'ev *et al.* (1979) and Cleveland *et al.* (1981) (Cleveland and Havercroft, 1983; Cleveland and Kirschner, 1982). Ben Ze'ev *et al.* (1979) demonstrated that colchicine, which depolymerizes microtubules and raises the level of tubulin, causes a cessation of tubulin synthesis by inhibiting the formation of new tubulin mRNA of cultured fibroblast cells. Similar conclusion was drawn by Cleveland and co-workers using immunoprecipitation and chicken  $\alpha$  and  $\beta$  tubulins cDNA probe to monitor the cellular response of several cell types to a wide range of antitubulin drugs. Thus, it has been postulated that in virtually all higher eukaryotic cells there is an autoregulatory mechanism which apparently acts to maintain a specific level of depolymerized tubulin subunits and responds rapidly to lower an elevated level of unpolymerized subunits and the effect is possibly at the level of transcription process. However, the synthesis of tubulin proteins in chicken fibroblasts does not respond to microtubule depolymerizing drugs in the same manner as cell lines from mammalian species, though the mRNA half life appears to be short (Cleveland *et al.*, 1981). Recently Cleveland and Havercroft (1983) have demonstrated that the rates of tubulin mRNA synthesis were essentially unchanged in isolated nuclei from CHO cells whether the cells were treated with colchicine or not. Thus, autoregulatory control of tubulin mRNA is not mediated through the regulation of transcription process *per se*. Alternatively this apparent regulation of tubulin mRNA by depolymerized tubulin might be due to inhibition of proper tubulin mRNA processing and/or transport from the nucleus. In this connection an interesting observation may be mentioned where it has been shown that nuclear matrix, nuclear envelope and cytoplasmic skeletal elements appear to be involved in maturation, transport and decay of mRNAs in general (Muller *et al.*, 1983). An interaction between the microtubules and the nuclear pore complex including the nucleoside triphosphatase might play a crucial role in the apparent autoregulatory control of tubulin mRNA synthesis.

In addition to regulation in response to unpolymerized tubulin levels, tubulin genes are differentially expressed during development. In *Drosophila melanogaster* there are four  $\alpha$  tubulin genes, all located at different sites on the third chromosome (Kalfayan and Wensink, 1981). Each gene yields characteristic mRNA levels at different stages of development as judged by RNA blot analyses using 3'-subcloned probes (Kalfayan and Wensink, 1982). At least two human  $\beta$  tubulin genes each specifying a distinct isotype, are expressed in HeLa cells, and the 2.6 kb mRNA band appears to be a composite of at least two comigrating  $\beta$  tubulin mRNAs (Hall *et al.*, 1983).

### Cloning and organization of tubulin genes

Tubulin gene from a variety of species has been cloned and sequenced (Cleveland, 1983) in a very short span of time. The first report of tubulin cDNA cloning starting from partially purified chick brain mRNA came from the laboratory of Kirschner (Cleveland *et al.*, 1980). The authors adopted the most widely used strategy for cDNA cloning. Tubulin mRNA which was purified by sucrose density gradient centrifugation and confirmed by its *in vitro* translated product, was transcribed by reverse transcriptase and then by DNA polymerase I. After S1 nuclease treatment, the double stranded cDNA was tailed at the 3'-end with cytosine residue using terminal polydeoxynucleotidyl transferase.



The sizing of cDNA (1000 to 2500 base pairs) was monitored and these species were annealed to plasmid pBR 322 that was linearized at the *pst*-1 restriction site and tailed with guanosine residues. Transformation of *Escherichia coli* with this recombinant DNA containing putative  $\alpha$  and  $\beta$  tubulin gene was performed according to the method of Goodman and MacDonald (1979). Selection of colonies was done by the colony hybridization technique (Grunstein and Hogness, 1975) using nick translated double stranded cDNA derived from enriched mRNA for tubulin. Hybrid plasmids containing cDNA sequences complementary to either  $\alpha$  or  $\beta$  tubulin were further identified by hybrid selected translation and identities of both  $\alpha$  and  $\beta$  tubulin clones have been confirmed by DNA sequencing. A similar approach was also used to prepare cDNA clone of  $\alpha$  and  $\beta$  tubulin genes in *Chlamydomonas* (Silflow and Rosenbaum, 1981). *Drosophila*  $\alpha$  tubulin clones were isolated by screening genomic DNA library using nick translated double stranded cDNA derived from purified mRNA specific for *Drosophila* tubulin (Kalfayan and Wensink, 1981). Tubulin genes have been cloned from human, rat, sea urchins, *Trypanosomes*, *Leishmania* and yeast using chicken brain cDNA as probe to screen either genomic library or cDNA library of respective species under appropriate stringent condition of hybridization to select colonies for  $\alpha$  or  $\beta$  tubulin (Cowan et al., 1981; Lemischka et al., 1981; Grinzburg, et al., 1981; Alexandraki and Ruderman, 1981; Thomashow et al., 1983; Landfear et al., 1983; Neff et al., 1983). Recently, the tubulin sequence complexities and their corresponding genomic organisation have been studied in a variety of species (Cleveland 1983).

*Chlamydomonas* gene organisation and expression have been studied by two groups of workers using cloned  $\alpha$  and  $\beta$  tubulin genes (Silflow and Rosenbaum, 1981; Brunke et al., 1982a,b) who demonstrated the existence of atleast two  $\alpha$  tubulin and two  $\beta$  tubulin genes in this organism. Hybridization experiments further suggest that during tubulin induction four tubulin mRNA of discrete sizes are produced (two  $\alpha$  and two  $\beta$  tubulin specific mRNA). *Chlamydomonas* cells contain several types of microtubules, including those found in the flagella, basal body, mitotic apparatus and cytoskeleton. Eventually it should be possible to determine whether all tubulin genes are expressed in this organism and to determine the type of microtubule(s) in which each gene product is found.

Alexandraki and Ruderman (1981) have analysed the multiplicity, heterogeneity and organization of the genes encoding the  $\alpha$  and  $\beta$  tubulins in the sea urchin, *Lytechinus pictus* by using cloned cDNA and genomic tubulin sequences. Hybrid selection performed at different stringency demonstrated the presence of several heterogeneous, closely related tubulin mRNA, suggesting the existence of heterogeneous  $\alpha$  and  $\beta$  tubulin genes. Hybridization analysis indicated that there are atleast 9 to 13 sequences for each of the two tubulin gene families per haploid genome.  $\alpha$  Tubulin genes and  $\beta$  tubulin genes are not found to be linked but in contrast, some genes within the same family are separated or dispersed. Exact number of functional genes of  $\alpha$  and  $\beta$  tubulin could not be ascertained out of a large number of tubulin genes estimated but the existence of atleast three different  $\alpha$  tubulin mRNAs which have considerable divergence in the 3'-nontranslated regions, could be demonstrated.

Recently, cDNA and genomic clones containing  $\alpha$  and  $\beta$  tubulins have been constructed from *Trypanosome brucei* (Thomashow et al., 1983; Seebach et al., 1983). Both groups of workers established that in contrast with the dispersed organization of

tubulin genes in other organism, Trypanosome  $\alpha$  and  $\beta$  tubulin genes are physically linked and clustered in tandem repeats of approximately 13–17 copies per haploid genome of alternating  $\alpha$  and  $\beta$  tubulin sequences. This arrangement may facilitate coordinate expression of the  $\alpha$  and  $\beta$  tubulin subunits in species where cycles of polymerization and depolymerization are major features of the cell cycle. The arrangement of developmentally regulated  $\alpha$  and  $\beta$  tubulin genes has been studied in the parasitic protozoan, *Leishmania enriettii* by using southern blot hybridization analysis (Landfear *et al.*, 1983). The  $\alpha$  tubulin genes occur in a tandem repeat whose monomeric unit may be represented by a 2-kilobase *pst*-1 fragment. Similarly, the  $\beta$  tubulin genes probably occur in a separate tandem repeat consisting of approximately 4-kilobase units unlinked to the tubulin repeats. In contrast to multiple  $\alpha$  or  $\beta$  tubulin genes found in all the organisms so far analysed, the lower eukaryote *Saccharomyces cerevisiae*, having simple nonmotile life cycle has only one  $\alpha$  and one  $\beta$  tubulin each in a haploid genome (Neff *et al.*, 1983). Presence of an unique tubulin gene in the yeast genome was shown by complementation of a benomyl resistant conditional-lethal mutation which carries mutation at the gene(s) specifying  $\beta$  tubulin with a cloned DNA fragment from yeast homologous to a chicken  $\beta$  tubulin cDNA and which has an essential function in yeast. In *Aspergillus nidulans*, structural gene for  $\beta$  tubulin was found to be situated at benomyl binding site and benomyl resistant mutants sometimes exhibit an altered  $\beta$ -tubulin protein (Sheir-Neiss *et al.*, 1978) and suppressor mutation of revertant of temperature sensitive *ben A* (benomyl resistant) mutant strain has been identified to be a structural gene mutation for  $\alpha$  tubulin in *Aspergillus nidulans* (Morris *et al.*, 1979). In rats and humans there are about 15 copies, well dispersed, each of  $\alpha$  and  $\beta$  tubulin sequences (Lemischka and Sharp, 1982; Cleveland *et al.*, 1980) and in chicken 4–5 copies each of these sequences (Cleveland *et al.*, 1980; Lopata, *et al.*, 1983), have been reported. Further, analysis of an expressed human  $\beta$  tubulin gene shows four coding regions of 57, 109, 113 and 1053 bp. The size of these exons is also similar to those in a second expressed human  $\beta$  tubulin gene (Cowan *et al.*, 1981; Lee *et al.*, 1983). This similarity in exon structure also extends to the exons of four expressed  $\beta$  tubulin genes of chicken (Lopata *et al.*, 1983). The size of the intervening sequences however, is highly variable. This suggests that sequences in exons in tubulin genes are well conserved while those in introns are not.

The tubulin gene system particularly *Chlamydomonas reinhardtii* shows rapid and coordinate induction of mRNA synthesis. Sequence analyses of those clustered genes reveal a short consensus sequence of 16 bp: [GCTC(G/C) AAGGC(G/T)(G/C) – (C/A)(C/A)G] just upstream of TATA box (Brunke *et al.*, 1984). In general, the putative regulatory elements within a particular gene set including developmentally and homonally controlled genes (Cochet *et al.*, 1982) and tubulin gene are similar but not identical (9–24 bp) and are present more than once in the upstream of TATA box. A summary of the sequence complexities of tubulin and the genomic organization in a variety of species is given in table 2. It is abundantly clear that the tubulin genes are present in large numbers in certain species and no significant correlation as a function of evolution could be established. The tubulin genes of most species thus examined constitute dispersed multigene families (Cleveland, 1983). The analysis of tubulin multigene family in different cases has shown that many of these sequences are pseudogenes (Lee *et al.*, 1983; Lemischka and Sharp, 1982).

Table 2. Summary of tubulin gene sequences in various species.

Species		No. of subunit gene per haploid genome	No. of functional genes	Sequence organiza- tion	References
Trypanosomes	$\alpha$	13-17	—	Tandemly duplicated	Thomashow <i>et al.</i> (1983).
	$\beta$	13-17	—	as $\alpha/\beta$ pairs	
Leishmania	$\alpha$	7-15	—	Clustered $\alpha$ 's	Landfear <i>et al.</i> (1983).
	$\beta$	7-15	—	Clustered $\beta$ 's	
Yeast	$\alpha$	1	1	Single gene	Neff <i>et al.</i> (1983).
	$\beta$	1	1	Single gene	
Chlamydomonas	$\alpha$	2	2	Dispersed	Silflow and Rosenbaum (1981)
	$\beta$	2	2	Dispersed	Brunke <i>et al.</i> (1982b).
Sea urchin	$\alpha$	15	—	Dispersed	Alexandraki and Ruderman (1981).
	$\beta$	15	—	Dispersed	
Drosophila	$\alpha$	4	4	Dispersed	Sanches <i>et al.</i> (1980), Kalfayan and Wensin (1981, 1982), Mischke and Pardue (1982), Raff <i>et al.</i> (1982).
	$\beta$	4	4	Dispersed	
Chicken	$\alpha$	4-5	4	Dispersed	Cleveland <i>et al.</i> (1980), Lopata <i>et al.</i> (1983).
	$\beta$	4-5	4	Dispersed	
Rat	$\alpha$	15-20	2	Dispersed	Lemischka <i>et al.</i> (1981), Ginzberg <i>et al.</i> (1981), Bond and Farmer (1983).
	$\beta$	15-20	2	Dispersed	
Human	$\alpha$	15-20	2	Dispersed	Hall <i>et al.</i> (1983).
	$\beta$	15-20	3	Dispersed	

### Tubulin pseudogenes and evolutionary significance

As has been mentioned earlier, the human tubulin gene family contains a number of pseudogenes thereby increasing the multiplicity of genes. In the case of the human  $\beta$  tubulin family, of the ten genomic sequences analyzed (Wilde *et al.*, 1982; Hall *et al.*, 1983; Lee *et al.*, 1983), seven are pseudogenes and the remaining three sequences represent functional genes. These pseudogenes contain multiple deletions and/or in frame translation termination codons within the exon sequences. Two of these seven are traditional pseudogenes that contain intervening sequences. The other five are of novel type to the extent that (i) each sequence lacks intervening sequences, (ii) downstream from the AATAAA consensus signal for poly(A) addition each carries a long coded tract of A residues and (iii) the entire sequence is flanked by a short direct repeat of 10-15 base pairs. It seems, therefore, that this type of pseudogenes originates by a reverse transcription event in which a mature mRNA is copied into DNA. That the pseudogenes are, however, discovered for a number of other genes beside tubulin gene suggests that this is a common feature in eukaryotic cell. Pseudogenes have also been described in the rat  $\alpha$  (Lemischka and Sharp, 1982) and human  $\alpha$  tubulin gene (Lee *et al.*, 1983). It is predicted that multigene families whose expression occur in the germline cells of higher vertebrates are likely to contain members of the processed type. In that

case similarities between the functional and processed pseudo human  $\beta$  tubulin gene are moderate, but an exclusive homology between 3'-untranslated regions is discernible (Hall *et al.*, 1983). Thus extensive homology between 3'-untranslated regions implies a close evolutionary relationship. In pseudogenes, mutations are found to be random since these are not subject to selective pressure. The functional gene, however, can acquire the changes that are silent (*i.e.* no amino acid change). Therefore, changes in the third codon position represent the cumulative effects of evolutionary drift in both functional and pseudogenes. Assuming that about half of the third base differences are due to changes in human  $\beta$  tubulin pseudogene and a rate of divergence of 0.7% per  $10^6$  years for neutral mutations (Perler *et al.*, 1980), this pseudogene may be estimated to have originated approximately  $4.4 \times 10^6$  years ago (Hall *et al.*, 1983). It is apparent that these integrated transcripts (pseudogenes) retain many of their original features for millions of years and might be recruited, in whole or in part, for the generation of new functional sequences.

## Conclusion

Microtubules are present in most eukaryotic cells where they fulfill diverse functions very vital to the existence of the cell. Until recently, tubulin research has been restricted exclusively to protein isolated from mammalian brain tissue. It is becoming increasingly apparent that, despite its conserved nature, tubulin exists as a family of proteins. As the range of organisms from which tubulin has been isolated broadens, the extent of diversification of the protein becomes clearer. Eukaryotic microorganisms are found to be particularly useful in revealing the heterogeneity of tubulins. It is interesting to study as to how the cells control differential assembly and dissociation of microtubules with the existence of different isotypes of tubulin subunits. The question also arises how to identify functionally different microtubules at specific stage of cell cycle and cell growth? In fact, different isotypes of tubulins have been identified in cytoplasmic pool, in flagella and in the pellicle of certain eukaryotic microorganisms. It will definitely be interesting if heterogeneity of tubulins from the spindle apparatus can be established.

That multiple tubulin genes are expressed differentially as a function of differentiation is now established. Whether each polypeptide produced is truly functionally distinct remains to be answered. Autoregulatory mechanisms of control of expression of  $\alpha$  and  $\beta$  tubulins have been well documented by the experimental results that colchicine, which depolymerizes microtubules and raises the level of free tubulin causes inhibition of tubulin synthesis by lowering tubulin mRNA production. However, this autoregulatory control of tubulin mRNA seems not to be mediated through the regulation of transcription process *per se*, instead, tubulin mRNA processing and/or transport from the nucleus might be the cause for this regulation. The question arises as to how tubulin exerts its effects on the processing and/or transport of tubulin mRNA. Considering that nuclear matrix, nuclear envelope and cytoskeletal elements might be involved in maturation, transport and decay of mRNAs in general, it would be interesting to find out any interaction of these components on the autoregulatory control of tubulin mRNA synthesis.

For what reason do the cells synthesize new tubulin for the flagella during differentiation inspite of the presence of abundant amount of tubulin? What leads to the expression of flagellar tubulin genes and what causes the disappearance of flagellar tubulin mRNA as the differentiation is completed? How, utilization of flagellar tubulin is related to its synthesis? These are the questions yet to be resolved.

Several antitumour drugs such as colchicine and its analogues, podophyllotoxin and vinca alkaloids inhibit mitosis and other cellular functions by specifically binding to tubulin and inhibiting its assembly into microtubule. The number of other chemical compounds which bind specifically with the tubulin particularly from eukaryotic microorganisms and plants has ever been increasing. However, data on the physico-chemical characteristics of the binding of these compounds except colchicine with tubulin are still limiting to propose any unifying concept for their binding with tubulin. It might turn out to be interesting to use these chemical compounds to delineate the different classes of microtubules and/or different populations of tubulins within a single microtubule.

The tubulin genes except the yeast gene are organized in multigene families. These are either tandemly arranged, clustered or well dispersed. The multigene family encoding tubulin proteins is of particular interest because, whereas numerous lines of evidence point to conservation of tubulin proteins, different species possess a broad range of tubulin like sequences. Although the coding regions of tubulin genes are rigidly conserved, the untranslated regions including the introns are not. Interestingly, the analysis of tubulin multigene families in different species has shown that many of these sequences are pseudogenes. Information on the sequences of these pseudogenes, both  $\alpha$  and  $\beta$ , suggest that most of these were derived *via* an mRNA intermediate. The accumulation of genetic lesions and the occurrence of oligo A tracts in intronless pseudogenes indicate that they are functionless and therefore not subject to selective pressure. The question then arises how these pseudogenes are retained? Since the pseudogenes retain many of the features of expressed genes it is possible that the generation of new functional sequences might arise out of them. In any case, the precise relationship between genetic complexity and microtubule function has not yet been elucidated.

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## Nutrients in the shadow-nutrients of substance

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**Abstract.** While the dietary importance of proteins, essential fatty acids, vitamins and trace elements has been well recognised, the role of shadow nutrients, a class of metabolites, which are biosynthesized in the body and serve vital functions, such as lipoic acid, choline, inositol, taurine and carnitine, has not been adequately appreciated. There are reasons to believe that during infancy and in ageing, biosynthesis of these metabolites may be limited. The objective of this review is to highlight the essentiality of these nutrients and the need for their supplementation in the diets of infants and in elderly people. Provision of shadow nutrients where the necessary biosynthetic machinery might not have developed to full stature or might have slowed down, is a new concept in nutrition which needs attention.

**Keywords.** Shadow nutrients; lipoic acid; diabetes; choline; acetyl choline; neuronal deficiency; inositol; taurine; carnitine; infant nutrition.

### Introduction

It is well recognised that certain essential amino acids, vitamins, fatty acids and trace elements are needed in the diet (Olson, 1978). It is often taken for granted that the mere provision of these essential nutrients in the diet, generates through biosynthesis all of the nutrients and intermediary metabolites necessary for normal health. Examples of these subsidiary nutrients or shadow nutrients are: nicotinic acid from tryptophan, choline from methionine and ethanolamine; vitamin B-12, and biotin endogenously made available by intestinal microorganisms in the gut, carnitine from lysine, inositol from glucose, lipoic acid from arachidonic acid and methionine and taurine from methionine. While these 'shadow' nutrients are produced in sufficient quantities under conditions of normal health, it is known that the physiological availability or biosynthesis of some of these nutrients is diminished in infancy, during ageing or when health is impaired. This is true not only of metabolic intermediates from nutrients, but also of neurotransmitters like serotonin, dopamine, epinephrine and acetylcholine formed from precursors such as tryptophan, tyrosine and choline (Wurtman, 1981). In this review we would like to highlight the nutritional importance of some of these factors such as lipoic acid, choline, taurine, inositol and carnitine.

The diet supplied to infants should be designed to bridge the nutritional gap between total intra-uterine dependence and complete extra-uterine independence and as such

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Abbreviations used: PD, Pyruvate dehydrogenase; PC, pyruvate carboxylase; CoA, coenzyme A; AcCoA, acetyl CoA; AcAcCoA, acetoacetyl CoA; Ac.ch., acetyl choline; CAT, carnitine acyltransferase.

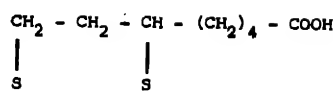
should contain easily digestible carbohydrates (lactose) and assimilable protein (non-coagulable protein) and easily digestible fat (triglycerides containing medium chain fatty acids and arachidonic acid). Further, due to the higher extra cellular fluid content, the lower ability of an infant's renal system to produce urine of increased specific gravity, the decreased renal clearance capacity and the incomplete development of the microsomal system for detoxification, infants need milk to graduate them to adult levels of absorption, digestion, assimilation and excretion. Histidine is essential in infants whereas in adults, it could be biosynthesized (Snyderman *et al.*, 1959, Holt and Snyderman, 1961). Other shadow nutrients which may be limiting in infancy are taurine and carnitine. There is danger in considering the infant as a miniature adult in terms of nutrition and metabolism.

Similarly, during ageing the metabolic availability of shadow nutrients such as lipoic acid, choline and inositol may be decreased either due to reduced rates of synthesis or due to the reduced caloric intake and consequent reduction in the ingestion of essential ingredients (McGandy *et al.*, 1966, Exton-Smith, 1980, Zeisel *et al.*, 1980). There is considerable evidence indicating deceleration during ageing of certain metabolic capabilities, such as neuronal acetyl choline synthesis and of inositol from glucose. (Campling and Nixon, 1954; Hauser, 1963; Davies and Maloney, 1976).

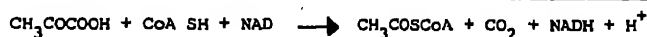
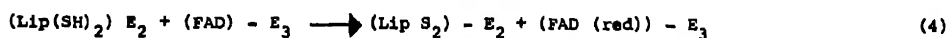
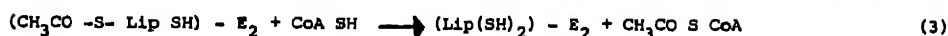
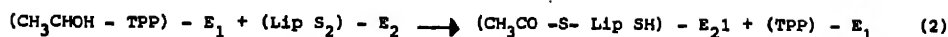
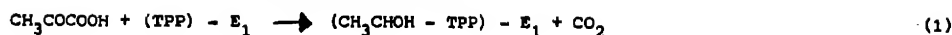
### Lipoic acid

Lipoic acid (figure 1) has long been recognised as a vital cofactor in the enzyme complexes that catalyse the oxidative decarboxylation of  $\alpha$ -keto acids such as pyruvic,  $\alpha$ -ketoglutaric and branched chain  $\alpha$ -keto acids formed during the catabolism of branched chain amino acids. Recently the decarboxylation of glycine has been shown to require lipoic acid (Fujiwara *et al.*, 1979). A dietary requirement for lipoic acid in animals has not been established, nor has a systematic estimation of the lipoic acid content in animals during ageing been done. The coenzymic role of lipoic acid in transacylation reactions involved in the oxidation of pyruvate is presented in figure 1.

Pyruvate is at the centre of metabolic disposition of substrates from the utilisation of proteins and carbohydrates and pyruvic dehydrogenase (PD) (EC 1.2.4.1) is crucial for the complete oxidation of glucose and for lipid biosynthesis from glucose (Jungas, 1970, 1971; Halperin, 1970). PD exists in a catalytically active (dephosphorylated) and an inactive (phosphorylated) form. Though the content of PD is the same in normal and diabetic livers, a larger proportion of the enzyme is in its inactive state in streptozotocin induced diabetes in rats (Weinberg and Utter, 1980). Similar reductions in active PD has been reported in perfused rat heart in alloxan diabetes (Kerbey *et al.*, 1976). Administration of insulin restored the PD activity to normal levels (Hughes *et al.*, 1980). We have found that administration of lipoic acid, like insulin treatment increases PD activity in the livers of both normal and diabetic rats (table 1). From table 2 it can be seen that blood pyruvate levels in alloxan diabetic rats are about 60% higher than normal and administration of lipoic acid reduces the elevated blood pyruvate in diabetic rats to near normal values in 60 min. We have previously shown that biochemical abnormalities such as hypoglycemia, ketonemia, reduction in liver glycogen and impaired incorporation of 2-[ $^{14}\text{C}$ ]-acetate into fatty acids in alloxan



Lipoic acid

Pyruvate oxidation $\text{E}_1$  = Pyruvate dehydrogenase (PD)

NAD = nicotinamide adenine dinucleotide

 $\text{E}_2$  = dihydrolipoyl transacetylase

FAD = flavin adenine dinucleotide

 $\text{E}_3$  = dihydrolipoyl dehydrogenase

TPP = thiamine pyro phosphate

Lip  $\text{S}_2$  and Lip  $(\text{SH})_2$  = oxidised and reduced lipoic acid

CoA SH = Coenzyme A

Stepp et al. (1981)

Figure 1. Lipoic acid.

Table 1. PD activity in normal and diabetic rat livers.

Rats	PD activity n mol/min/mg protein
Normal	2.7 ± 0.28
Normal + lipoic acid	4.3 ± 0.19 <sup>a</sup>
Normal + insulin	4.7 ± 0.42 <sup>a</sup>
Diabetic	1.26 ± 0.29 <sup>a</sup>
Diabetic + lipoic acid	2.40 ± 0.30 <sup>b</sup>
Diabetic + insulin	2.66 ± 0.10 <sup>c</sup>

All the determinations are means of 6 animals ± S.E.

<sup>a</sup> Significantly different from normal at  $P < 0.01$ <sup>b</sup> Significantly different from diabetic at  $P < 0.05$ <sup>c</sup> Significantly different from diabetic at  $P < 0.01$ 

diabetic rats were brought to near normal levels by the oral or intraperitoneal administration of lipoic or dihydrolipoic acid (Natraj *et al.*, 1984). Lipoic acid content in diabetic livers is markedly reduced as compared to its amount in normal liver (Natraj *et al.*, 1984).

In diabetes, along with impairment of pyruvate oxidation (Vilee and Hastings, 1949),

Table 2. Pyruvate levels in blood.

	Pyruvate levels $\mu$ mol/litre
Normal (4)	85.4 $\pm$ 16.4
Diabetic (5)	137.4 $\pm$ 3.7*
Diabetic + lipoic acid after 60 min (5)	93.1 $\pm$ 17.2**

Numbers in the parenthesis indicate the number of animals. The values are expressed as mean  $\pm$  S.E.

$\alpha$ -Lipoic acid (sodium salt, 10 mg/100 g) was injected intraperitoneally as a solution in 0.1 ml water.

\* Significantly different from normal  $P < 0.05$ .

\*\* Significantly different from diabetic  $P < 0.05$ .

increased gluconeogenesis also occurs (Felig *et al.*, 1970; Felig, 1975). Enhanced activities of specific gluconeogenic enzymes including cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-bisphosphatase (EC 3.1.3.11), glucose 6-phosphatase (EC 3.1.3.9) and pyruvic carboxylase (EC 6.4.1.1) have been reported in experimental diabetes (Prinz and Seubert, 1964; Filsell *et al.*, 1969; Wilmhurst and Manchester, 1970; Chang and Schneider, 1971; Weiss *et al.*, 1971). Mitochondrial pyruvic carboxylase (PC) catalyses and initiates gluconeogenesis. Weinberg and Utter (1980) using immunochemical techniques, demonstrated that in the livers from streptozotocin induced diabetic rats, pyruvic carboxylase activity was twice that in normal rat livers when expressed in terms of DNA or body weight; this increase was shown to be due to significant increases in the rate of synthesis of the enzyme; whereas the rates of synthesis and degradation of PD were not affected. The flux of pyruvate carbon atoms through PD and PC and the regulation of these enzyme activities are shown in figure 2. It is interesting to note that the high production of acetyl (Ac)-coenzyme A-(CoA) and generation of aceto-acetate in diabetes appears to arise not from the oxidative decarboxylation of pyruvate but through the increased oxidation of fatty acids (Randle *et al.*, 1966).

It has been proposed that the increased oxidation of fatty acids generates excess AcCoA and acetoacetyl CoA (AcAcCoA) which acylate the lipoic acid residues of PD. Acylated lipoic acid has been shown to activate PD kinase and thus bring about inhibition of PD (Cate and Roche, 1979). The respective metabolic roles of PD and PC in various organs are illustrated in table 3. It will be seen from the table that PC is crucial for gluconeogenesis, whereas PD is essential for energy production. In diabetes, the energy production occurs through the oxidation of fatty acids and it is well recognised that the respiratory quotient is low due to abnormal fatty acid oxidation (Randle, 1976). In gluconeogenic organs such as the kidney, PC > PD, but in energy consuming organs like the heart PD > PC. Whereas in the normal liver PD and PC are finely balanced, in the diabetic liver PC > PD due to the increased demand for glucose (Randle *et al.*, 1977).

A decreased glucose tolerance is common in a majority of people during ageing. The diminution in the levels of lipoic acid in the liver of diabetic rats and the effectiveness of

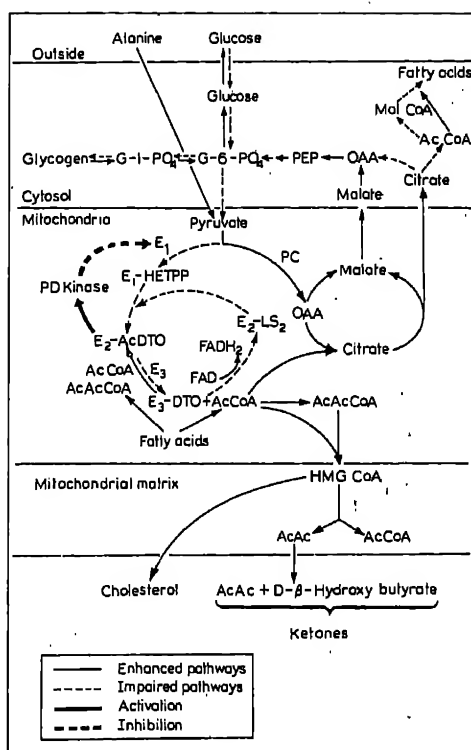


Figure 2. Metabolic map in diabetes.

Table 3. Regulation of PD and PC in various organs.

PD and PC are mitochondrial enzymes
PD is required for energy production
PC is required for gluconeogenesis
PD + PC is required for fatty acid biosynthesis
In heart PD > PC (energy consuming organ)
In kidney PC > PD (gluconeogenic organ)
In adipose tissue PD = PC (fatty acid biosynthetic)
In liver PD = PC (all the above three)
In diabetic liver PC > PD (gluconeogenic)

From Randle *et al.* (1977).

dietary lipoic acid in restoring most of the biochemical abnormalities in diabetes led us to consider whether the biosynthesis of lipoic acid is impaired during ageing/diabetes. Though lipoic acid is a ubiquitous component in all aerobic organisms and animals, most tissues and microorganisms contain only minute quantities of this material. Lipoic acid is biosynthesized in *Escherichia coli* from octanoic acid, hydroxy octanoic acid and more efficiently from thiooctanoic acid (White, 1980a,b; 1981). Carreau *et al.*



Table 4. Incorporation of 1-[ $^{14}\text{C}$ ]-labelled compounds into liver fat and lipoic acid.

Labelled precursor	Rats	Amount perfused in liver cpm ( $\times 10^6$ )	Radioactivity incorporated in fat cpm ( $\times 10^6$ )	per cent	Radioactivity recovered in TLC fraction cpm ( $\times 10^3$ )	per cent	Radioactivity recovered in HPLC-lipoic acid cpm ( $10^3$ )	per cent
Arachidonic acid	N	1.9	1.06	55.6	16.6	0.87	2400	0.125
	D	1.7	0.690	40.3	6.84	0.398	560	0.032
Octanoic acid	N	3.2	NE	NE	23.56	0.73	3520	0.109
	D	4.3	0.34	7.9	8.05	0.185	508	0.012
Linoleic acid	N	8.18	2.59	31.7	48.20	0.59	6650	0.081
	D	4.8	0.89	18.4	0.59	0.012	ND	ND
Palmitic acid	N	8.0	0.15	1.94	0.10	0.001	ND	ND

Livers were perfused with the labelled precursor for 1 h and incubated for 3 h at room temperature. Lipoic acid was isolated by extraction followed by TLC. The TLC fraction was further purified by HPLC.

NE—not estimated. ND—not detectable.

(1977) have shown that linoleic acid and to a smaller extent oleic acid, act as precursors for lipoic acid biosynthesis in the rat. Further, the subcellular location of lipoic acid biosynthesis has been shown to be the microsomal fraction in rat liver (Spoto *et al.*, 1982). However, as shown in table 4, in animal tissues arachidonic acid appears to be the most immediate precursor followed by linoleic acid, though octanoic acid incorporated into lipoic acid is unlikely to be of physiological significance in view of the unlikely occurrence of free octanoic acid in body metabolism. The scheme of biosynthesis of lipoic acid from arachidonic acid is given in figure 3.

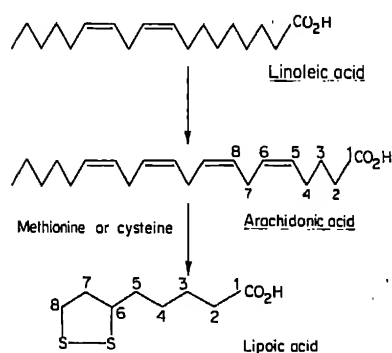


Figure 3. Biosynthesis of lipoic acid.

Further, methionine and cysteine have been shown to be equally effective as sulphur donors in the biosynthesis of lipoic acid (Dupre *et al.*, 1980). We have confirmed this finding in experiments with diabetic rats. We have further demonstrated that the biosynthesis of lipoic acid from linoleic acid is impaired in diabetes and administration of insulin enhances this conversion (table 5).

Table 5. Incorporation of 1- $^{14}\text{C}$ -linoleic acid into lipoic acid in presence of insulin.

Rats	Radioactivity (cpm)				Per cent incorporation	
	Amount perfused ( $\times 10^6$ )	Fat ( $\times 10^6$ )	Lipoic (TLC)	Lipoic (HPLC)	Fat	Lipoic (HPLC)
Normal	5.27	1.72	27,000	3,800	32.6	0.072
Diabetic	3.63	0.54	6,100	N.D.	14.4	N.D.
Diabetic + insulin	2.24	0.8	3,500	810	35.7	0.036

N.D.—not detectable

Livers were perfused with the labelled precursor for 1 h and incubated for 3 h at room temperature. In experiments with insulin, 0.25 i.u./ml of insulin was used in the perfusion medium. Lipoic acid was isolated and purified as described in table 4.

Essential fatty acids have an insulin sparing effect in diabetes (Houtsmuller *et al.*, 1981). Is this effect due to an increased biosynthesis of lipoic acid from linoleic acid?

To summarise, the beneficial effects of lipoic acid administration in diabetes have been recognised for some time (Pagliaro, 1956; Pagliaro and Furitano, 1956; Greco, 1957; Brusa and Serafini, 1958; Zueva, 1970). Our observations that lipoic acid acts

primarily by enhancing the utilisation of glucose for energy and fatty acid synthesis, open up the possibility of using this shadow nutrient as an adjunct in the diet in cases where the tolerance to glucose is reduced (*e.g.* ageing and diabetes).

## Choline

Choline, a quaternary ammonium base, is distributed ubiquitously in biological materials. It is present as an integral part of the membranes of all cells, as a constituent of lecithins, plasmalogens and sphingomyelins. Acetylcholine is responsible for the transmission of nerve impulses from presynaptic to postsynaptic fibres in the synapses of both the sympathetic and parasympathetic nervous systems. Choline is converted into acetylcholine by choline acetylase and is hydrolysed by acetylcholinesterase into choline and acetate.

A dietary requirement of choline was first demonstrated by Best and Huntsman (1932). Since then choline deficiency has been shown to cause haemorrhagic lesions in the kidney and fatty infiltration in the liver in rats and perosis in birds (Griffith and Wade, 1939; Jukes, 1940; Hartroft, 1955). From the voluminous literature on choline it is evident that it has a lipotropic effect in the liver and increases the formation and secretion of chylomicrons in the intestine (Tidwell, 1950; Frazer, 1978). Choline is biosynthesized in the body from precursors such as methionine and ethanolamine (figure 4) and lecithin *via* methylation of phosphatidyl ethanolamine (figure 5). Though

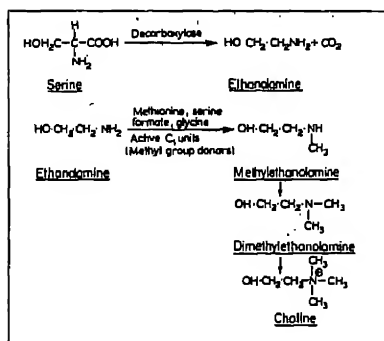


Figure 4. Biosynthesis of choline.

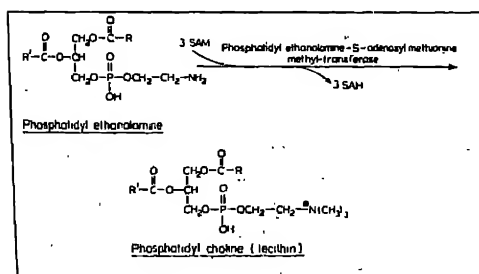


Figure 5. Biosynthesis of phosphatidyl choline.

a specific deficiency of choline has not been demonstrated in humans, in many conditions of liver dysfunction, the advantages of feeding choline or its precursors such as methionine have been well established (Kautch, 1973; Wallnofer and Hanusch, 1973).

The paradox, however, is that almost all animal cells have the biosynthetic machinery to synthesize choline from its precursors, thus rendering dietary supplementation of choline or its derivatives somewhat redundant. Further, lecithin (a phospholipid containing choline) is largely degraded in the intestine. Despite these observations, unequivocal evidence has been obtained demonstrating the beneficial effects of choline and lecithin administration in cases of tardive dyskinesia and Huntington's disease (Davis *et al.*, 1976; Tamminga *et al.*, 1977; Growdon *et al.*, 1978).

The hippocampus, a region of the brain known to be essential for the formation of new memories, has a particularly large number of cholinergic neurons. Based on the observation that muscarinic agonists such as physostigmine (inhibition of acetylcholinesterase) can improve memory acutely, choline and lecithin have been tried, with limited success, in patients with Alzheimer's type dementia and mild to moderate memory disorders (Etienne *et al.*, 1968; Signoret *et al.*, 1978). Further, there is recent evidence that the onset of senility is characterized by a decrease in the availability of acetyl choline (Ac.ch.) to the neuronal cells in the brain, which seems to be caused by the decreased availability of free choline (Gibson and Peterson, 1981). Since choline has been shown to diffuse across the blood brain barrier, treatments which raise serum choline such as ingesting choline rich foods would provide a way of increasing the supply to the brain.

It must be mentioned in this connection that Dysken *et al.* (1982) in a limited clinical study with patients with primary degenerative dementia failed to observe any benefit with the administration of lecithin, while earlier reports had claimed significant benefits with the administration of choline bitartrate (Fovall *et al.*, 1980; Dysken *et al.*, 1982).

Although lecithin has been recommended and even tried in clinical trials, the practicability of including upto 70–100 g/day of lecithin in the diet poses problems.

This raises the question as to what should be the preferred dietary additive for enhancing choline availability to the brain for the synthesis of acetylcholine? There is considerable evidence indicating a lack of equilibration between choline and lecithin in the body. (1) Choline deficiency and its attendant symptoms such as impaired growth, haemorrhagic kidneys and fatty liver occur in the face of apparently normal levels of lecithin in the liver and the whole organism (Menon and Lucas, 1961). Only the administration of choline helps to overcome these problems. (2) Phospholipase D (figure 6) is responsible for the hydrolysis of lecithin to free choline. Although an enzyme with phospholipase D like activity has been isolated and purified from rat brain, the contribution of this enzyme *in vivo* for the release of free choline from lecithin is yet to be established (Taki and Kaufer, 1981). (3) Phospholipase C releases phosphoryl choline from lecithin and sphingomyelin and is present in most animal tissues. The phosphoryl choline can be cleaved by alkaline phosphatase with the release of free choline. However, this method of free choline production would require very high levels of lecithin in the tissue (brain) which may be difficult to attain. (4) Lecithin in animals is biosynthesized *via* methylation of phosphatidyl ethanolamine (cephalin) rather than from phosphatidic acid and choline, and (5) Lecithin synthesis through phosphoryl choline (PC) and  $\alpha$ ,  $\beta$  diglyceride requires PC synthesis from free choline.

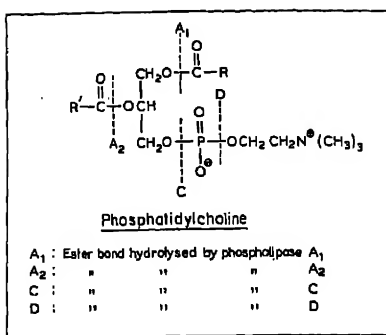


Figure 6. Phospholipases.

Thus PC is more involved in the synthesis of lecithin rather than being the source of bioavailable choline (Kornberg and Pricer, 1952; Kennedy and Weiss, 1956).

Further, there is no evidence indicating that the intake of egg yolk containing 0.9 % lecithin in humans in anyway protects them against neuropathology or dementing illnesses such as Alzheimer's disease or memory disorders associated with old age. However, both positive and negative clinical response to high doses of lecithin in Alzheimer disease and primary degenerative dementia have been reported (Etienne *et al.*, 1979). It may be stressed that here we are considering the beneficial effects on health of supplementing with small amounts, those cofactors which may be lacking in the diet and not the therapeutic treatment with choline or its derivatives (Dysken *et al.*, 1982; Karczmar, 1979).

From these considerations it would appear that in order to increase blood levels of choline one has to administer free choline or choline esters. Since free choline or even phosphoryl choline has a fishy odour and is not acceptable organoleptically for incorporation into foods, it is suggested that linoleate or palmitate esters of choline be used for this purpose.

## Inositol

Like choline, inositol (figure 7) is also well recognised as a vitamin for a number of organisms. In fact, the effectiveness of hexachlorocyclohexane in killing cockroaches is due to its inhibitory effect on inositol which is an essential nutrient for insects (Slade, 1945). It must be emphasized that there is a commonality in the requirements of vitamins amongst a broad phylum of organisms including bacteria, yeast, insects and mammals, and therefore even though a specific inositol deficiency state has not been reported in humans, there is no doubt about the need for inositol in human metabolism.

Phosphatidyl inositol, one of the major anionic phospholipids of mammalian cell membranes, undergoes stepwise phosphorylation to diphosphoinositide in synaptosomal fractions in the brain and to triphosphoinositide in erythrocytes and myelin membranes (Hawthorne and White, 1975). Hormones such as vasopressin cause rapid breakdown of polyphosphoinositides and may exert a primary role in generating messenger signals responsible for intracellular  $\text{Ca}^{2+}$  mobilization (Thomas *et al.*, 1983). Recent evidence suggests that polyphosphoinositides breakdown to phosphatidylinositol in a number of systems, including muscarinic stimulation of smooth muscle, nerve

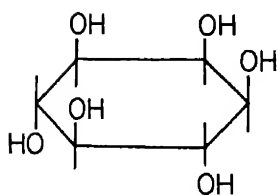
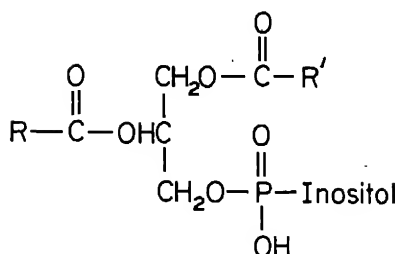
Myo-inositolPhosphoinositide

Figure 7. Inositol.

endings and parotid gland and thrombin addition to human platelets (Abdel-Latif *et al.*, 1977; Fischer and Argranoff, 1981; Weiss *et al.*, 1982; Agranoff *et al.*, 1983).

Phosphoinositides form 70% of the phospholipids of the myelin sheath which is a major insulating material in the human nervous system and caters to the brain's conflicting needs for compact size, complex circuitry, rapid signalling and modest use of energy. The brain is especially rich in inositol phosphatides (Herken *et al.*, 1958). There is evidence to suggest that the biosynthesis of inositol from glucose is decreased as animals grow older (Hauser, 1963). Even as lecithin does not contribute to the pool of free choline in the body, inositol from phytin does not seem to be available as free inositol for the maintenance of body functions.

Inositol has been shown to be a necessary vitamin for growth of mammalian cells (Eagle, 1956); stimulation of intestinal peristalsis, in plasma membrane shape regulation (Quist and Reece, 1980); and transport of cations across membranes especially for the triggering of nerves (Martin *et al.*, 1941; Hawthorne and White, 1975). Older nutrition literature has given a significant status to inositol in reducing cholesterol esters in the liver (MacFarland and McHenry, 1945, 1948) and it is likely that inositol might also promote reduction of blood cholesterol (Rajalakshmi *et al.*, 1960). Foods containing high amounts of inositol are yeast and tea.

### Taurine

Taurine (figure 8) is one of the major constituents of the free amino acid pool in all mammalian central nervous system tissues. In a mature brain it is usually exceeded in

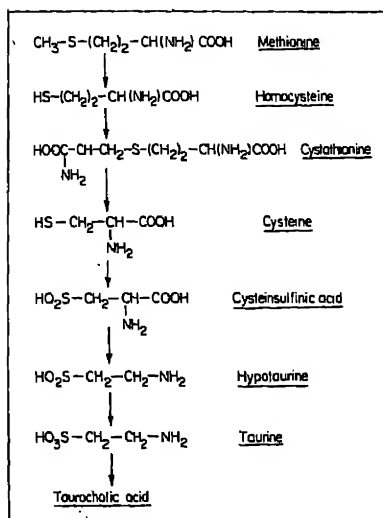


Figure 8. Biosynthesis of taurine.

concentration only by glutamic acid and in the developing brain, it is the free amino acid present in the greatest concentration. The diversity and magnitude of the functions accomplished or influenced by such a chemically simple and inert compound has only been emphasized in recent years (Jacobsen, 1980). The major physiological functions of taurine are (i) participation in bile conjugation, (ii) osmoregulation, (iii) neurotransmission and neuroregulation and (iv) membrane stabilization.

Taurine is a constituent of taurocholic acid excreted in the bile of carnivorous animals and man. Though taurine is ubiquitously present in most tissues of the body, recent evidence has surfaced on the role of taurine in moderating neurotransmission, in stimulating retinal metabolism (Mandel *et al.*, 1976; Schmidt *et al.*, 1976), and most importantly in preventing cardiac arrhythmia (Aker and Brody, 1976; Grosso and Bessler, 1976). Taurine has been shown to confer calcium and potassium stabilising capacity on the heart and the brain when circumstances of electrolyte depletion are present, thus ensuring cationic and membrane integrity (Aker and Brody, 1976). Taurine therapy has been efficacious in experimental models of epilepsy (Barbeau and Donaldson, 1973; Van Gelder *et al.*, 1975). There have also been reports of taurine depletion in cardiac ischaemia and the ameliorative effect of taurine treatment in such conditions (Huxtable and Bressler, 1974a,b). There is an increasing quantum of evidence that human infants fed casein-predominant synthetic formulations derived from bovine milk may become taurine depleted because the currently available products contain little or no taurine (Nayman *et al.*, 1979; Rigo and Senterre, 1977). In this context, it is interesting to note that recent studies have demonstrated that in infants fed human milk, bile acids predominantly conjugated with taurine during the first three months after birth, whereas in infants fed synthetic formulations the bile acids conjugated predominantly with glycine (Haber *et al.*, 1978; Watkins *et al.*, 1979). Because of its inhibitory neurotransmittary role in the brain and retina and because of its importance in the conjugation of bile acids with superior digestive and detergent

properties, there is some nutritional logic in introducing taurine as an additive in many vegetable products.

## Carnitine

Carnitine is a quaternary amine,  $\beta$ -hydroxy,  $\gamma$ -N-tri-methylaminobutyric acid, which is an important metabolite in mammalian tissues for the utilization of long chain fatty acids as a source of energy (Hoppel, 1982). Carnitine facilitates the transport of fatty acids from the cytosol across the mitochondrial membrane *via* the carnitine acyl-transferase (CAT) (EC 2.3.1.7) and translocase systems (figure 9) (Bremer, 1977). Carnitine is synthesized in both human liver and kidney from the essential amino acids lysine and methionine (figure 10) (Rebouche and Engel, 1980; Tanphaichitr and Broquist, 1973). Several other tissues can synthesize the immediate precursor of carnitine ( $\gamma$ -butyrobetaine) but lack the last biosynthetic enzyme which hydroxylates  $\gamma$ -butyrobetaine to form carnitine. Since cardiac and skeletal muscles cannot synthesize

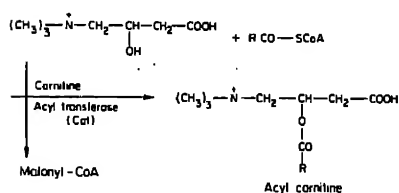


Figure 9. Fatty acid transport.

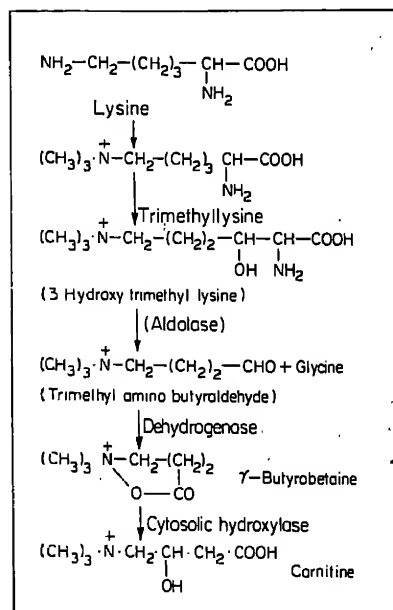


Figure 10. Biosynthesis of carnitine.



carnitine, it must be transported into these tissues from the blood stream (Borum, 1981).

The ability of the rat liver to synthesize carnitine from  $\gamma$ -butyrobetaine increases from low values in the foetus to adult values on the eighth day after birth. The newborn's requirement for carnitine appears to be adequately met by breast milk but there is no detectable carnitine in soya protein based formulations, nor is it added to solutions used in total parenteral nutrition.

The level of malonyl-CoA determines the rate of fatty acid synthesis, whereas the activity of CAT determines the rate of fatty acid oxidation. With high dietary carbohydrate, malonyl-CoA concentration and consequently, fatty acid synthesis increase, while fatty acid oxidation is suppressed by the inhibition of CAT (figure 9). In the fasting animal and in diabetes malonyl-CoA level drops, fatty acid synthesis declines and with lower malonyl-CoA levels CAT is no longer inhibited and therefore fatty acid oxidation increases (*Nutr. Rev.*, 1980a; McGarry and Foster, 1979; McGarry *et al.*, 1978; Broquist, 1982).

Further, a role for carnitine in the metabolism of branched chain keto acids produced by the catabolism of amino acids has been suggested (*Nutr. Rev.*, 1981). Muscle weakness and pathology in humans with accompanying myoglobinuria has been shown to be due to a relative deficiency of carnitine and can be treated with high carbohydrate feeding (*Nutr. Rev.*, 1979b). Low birth weight infants with impairment in the lipid and energy utilization may have subnormal carnitine levels in the blood (*Nutr. Rev.*, 1980b). There may therefore be a nutritional advantage in incorporating carnitine or carnitine containing foods such as meat or meat extracts in infant foods.

Some of these shadow nutrients may be synthesized in the animal body in marginal quantities at certain times in the life cycle. There may be an increased need for some of these nutrients to be provided specifically in the diets of the elderly or infants. If these nutrients are not biosynthesized adequately there would be a need for supplementing these exogenously in the diet. Such nutrients may include lipoic acid, choline, taurine, inositol and carnitine.

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## Differentiation of pathogenic amoebae: encystation and excystation of *Acanthamoeba culbertsoni*—A model

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**Abstract.** Differentiation into dormant cysts and vegetative trophozoites is an inherent character intimately associated with the life cycle and infectivity of pathogenic amoebae. In the case of human intestinal amoebiasis encystation and excystation are of immediate relevance to the process of transmission of the disease from healthy carriers to susceptible individuals. Using a pathogenic free living amoeba *Acanthamoeba culbertsoni* as a model, considerable progress has been achieved in understanding the mechanism and control of the process of differentiation. The turnover of the regulatory molecule cyclic 3':5' adenosine monophosphate is responsible for triggering the process of encystation. Amoebae bind effector molecules such as biogenic amines to a membrane localized receptor which itself resembles the  $\beta$ -adrenergic receptor of mammalian organisms. The activation of adenylate cyclase or inhibition of cyclic AMP phosphodiesterase maintain the dynamic intracellular cyclic AMP. The cytosol fraction of amoebae has a cyclic AMP binding protein. During encystation, enzymes needed for synthesis of cellulose and glycoproteins are induced. Control is exercised at transcriptional level and the process is subject to catabolic repression.

Excystation of mature amoebic cysts is mediated by glutamic acid and certain other amino acids by an as yet unelucidated mechanism. During excystation there is dormancy break, induction of depolymerizing enzymes viz. two proteases, a cellulase and a chitinase. The empty cysts or cyst walls are digested by these enzymes and their break down products are used for cellular growth. By invoking a flip-flop mechanism of repression and derepression some plausible explanation can be offered for the cascade of biochemical events that sets in when amoeba is 'turned on' to encystation or excystation.

**Keywords.** Amoeba; encystation; excystation; cyclic AMP.

### Introduction

The phylum protozoa includes many microorganisms of relevance to human health and the environment (table 1). Thus malaria, sleeping sickness, amoebiasis, giardiasis, leishmaniasis are all human afflictions caused by protozoa and represent the phenomenon of parasitism. On the other side we have in protozoons many examples of symbiosis or biological interdependence such as in the *Mixotricha paradoxa* a protozoon which lives in the gut of the Australian termite. *M. paradoxa* is covered by thousands of spirochetes. When the latter move, *M. paradoxa* is propelled along smoothly, its own flagella being used only for steering. Another classical example of symbiosis is the protozoon *Paramoecia bursaria* which derives its colouration from the microscopic algae of the *Chlorella* genus which it harbours. The malarial parasite *Plasmodium falciparum* uses an arthropod vector, the mosquito, and the human host to

Table 1. Protozoan diseases of man.

Amoebiasis	Intestinal, Hepatic, brain	<i>Entamoeba histolytica</i>	infective cyst in faeces, invasive trophozoite in intestinal and other tissues
Primary Amoebic	Central nervous system	<i>Acanthamoeba castellanii</i>	Free-living amoebae in water, soil and decaying vegetation, as cysts; trophozoites in tissues
Meningo-encephalitis		<i>Naegleria fowleri</i>	
Giardiasis	Intestinal	<i>Hartmannella</i> sp. <i>Giardia lamblia</i>	Cysts or trophozoites in faeces; trophozoites in deodermal drainage
Trichomoniasis	Caecum/colon	<i>Trichomonas hominis</i>	non-pathogenic sp.—only trophozoite known (no cystic stage) in diarrhoea
	Genito-urinary tract of women urethra of men	<i>Trichomonas vaginalis</i>	motile flagellate in the discharge (trophozoite)
Trypanosomiasis African (Sleeping sickness)	Blood, lymph nodes, cerebrospinal fluid	<i>Trypanosoma gambiense</i> <i>Trypanosoma rhodesiense</i>	infective mastigote stage in the salivary glands and mouth. <i>Glossina</i> sp. (tse-tse fly) inoculated into blood of man
American (Chagas' Disease)		<i>Trypanosoma cruzi</i>	infective metacyclic stage in the faecal droplet of the bug <i>Triatoma</i> contaminating its bite on conjunction mucous membranes. esp. of children.
Leishmaniasis	Visceral (Kala-azar) spleen, liver, lymph node or blood	<i>Leishmania donovani</i>	flagellate infective stage in the sand-fly, <i>Phlebotomus argentipes</i> , inoculated into blood by its bite, amastigote stage in spleen.
Malaria	Cutaneous (Oriental Sore) skin and mucous membrane of nose Blood and tissues (liver, spleen, etc.)	<i>Leishmania tropica</i> <i>L. brasiliensis</i> <i>Plasmodium vivax</i> (Benign tertian) <i>P. malariae</i> (Quartan) <i>P. falciparum</i> (Malignant tertian) <i>P. ovale</i> (ovale malaria)	as above, in <i>P. sergenti</i> in India, its bite ( <i>L. brasiliensis</i> ) in South America. Transmitted by bite of various species of mosquitoes (Anopheles) sporozoites in salivary glands (infective) and trophozoite, schizont and gametocyte in man
Balantidiasis	intestinal—causing dysentery	<i>Balantidium coli</i>	cysts of the ciliate in faeces of man, contaminating soil, water, vegetables hands etc. and ingested
Toxoplasmosis	body tissues (esp of <i>Toxoplasma gondii</i> the eye) intra-cellular chorio-retinitis, iritis, etc.	<i>Toxoplasma gondii</i>	cystic stages of the coccidian in faeces of cats, trophozoite (invasive stage) in tissues of man.

complete its differentiation process the crucial steps of which have defied so far all research endeavours.

Ciliated protozoons, parasitic and free living amoebae are among the unicellular organisms other than bacteria which biochemists have used extensively for elucidating the mechanism of the chemical reactions and control processes operating behind the scene in cellular differentiation. *Tetrahymena pyriformis* has been a good model for investigations into the molecular basis of ciliary movement, adaption to unfriendly environments and the phenomenon of rapid regeneration of tissues. The free living amoebae *Acanthamoeba* have been very useful in gaining understanding of the chemical processes of encystation and excystation. An account of recent work on amoebic differentiation is presented in this review.

### Definitions

The process by which amoebae lose their characteristic pseudopoidal movement and get endowed with a protective and impenetrable wall is called encystation. The resulting round bodies are called cysts. Cysts are immotile and remain metabolically inert as long as conditions are unfavourable for hatching. Once the appropriate environment for dormancy break is established, the cysts shed their coat and resume trophic or vegetative life by a process called excystation. Encystation could also be considered as a defensive posture adopted by the amoeba to overcome stresses imposed on it by a hostile environment. By analogy, excystation would constitute the emergence of the amoeba from a dormant to an active metabolic status. Transformation of trophic amoebae to dormant cysts and the reincarnation of the cysts as trophozoites (figure 1)

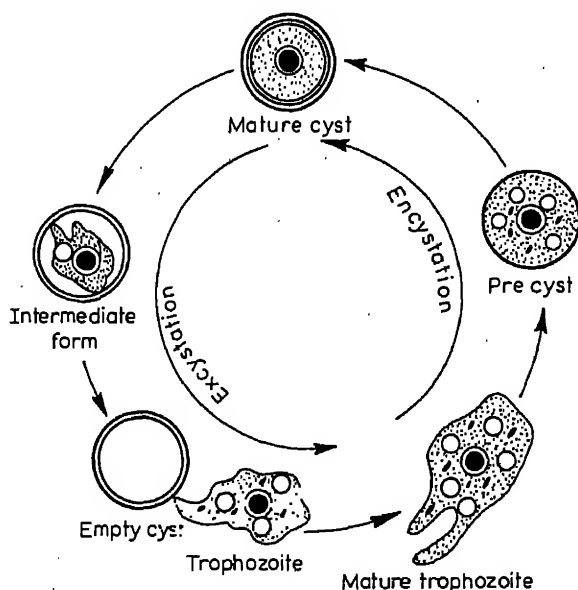


Figure 1. Schematic representation of differentiation in *A. culbertsoni*.

envisage cellular differentiation subject to many attendant control mechanisms (Trager, 1963; Willmer, 1963; Sussman, 1965; Wright, 1966; Krishna Murti, 1971, 1975).

### Differentiation and pathogenesis

Biochemical reactions mediated during differentiation are germane to the pathology of endemic amoebiasis caused by the human intestinal parasite *Entamoeba histolytica* and the pathology of amoebic encephalitis caused by a few strains of free living *Acanthamoeba*. With a high degree of endemicity in Egypt (85%), Equador (56%), Liberia (52%) and India (35%) and a global prevalence rate of 10% amoebiasis in its intestinal and hepatic manifestations is as yet an unsolved public health problem in the Third World (Freedman, 1958; WHO, 1969). Attempts to demonstrate a toxin-linked pathogenic mechanism have been unsuccessful. At present there are no immunological guide posts for devising preventive or prophylactic strategies excepting the environmental control of the source of infection. Acute attacks of amoebic dysentery are usually amenable to management by amoebicidal drugs, such as halogenated hydroxy quinolines, metranidazole, chloroquine or some antibiotics. In contrast, chronic amoebiasis associated with the harbouring of recalcitrant cysts continues to be a clinical problem (WHO, 1969). The management of infestinal amoebiasis is complicated by the fact that many healthy persons who have never suffered from amoebiasis are carriers of infective cysts.

Amoebic meningoencephalitis due to free living amoebae has been recognised as a distinct clinico-pathological entity (Fowler and Carter, 1965; Batt, 1966; Culbertson *et al.*, 1966). Pathogenic strains of *Naegleria* have been isolated from human cerebrospinal fluid (Carva and Novak, 1968) and implicated in fatal meningoencephalitis in man (Carter, 1968; Duma, 1972, 1980). The pathogenic potential of *Hartmannella culbertsoni* now christened as *Acanthamoeba culbertsoni* is manifest as cerebral (Kenny, 1971; Jager and Stamm, 1972; Robert and Rorke, 1973; Bhagwande *et al.*, 1975), respiratory (Eldridge, 1967) and eye infections (Naginton *et al.*, 1974; Visveswara *et al.*, 1975). Fatal meningoencephalitis has also been reported with *Acanthamoeba* (Carter *et al.*, 1981; Grunnet *et al.*, 1981). The ubiquitous distribution of free living amoebae in fresh water, sea water, the melt water of Antarctica, the latex of *Euphorbia*, soil, cultures of animal cell lines maintained in laboratories and even in samples of bottled mineral water in some regions adds yet another dimension *viz.*, environmental contamination, to the pathology associated with these organisms (Jahnes *et al.*, 1957; Culbertson, 1961; Chang *et al.*, 1962; Moore and Hlinka, 1968; Casemere, 1969; Peloux *et al.*, 1974; Singh, 1965; Visveswara and Balamuth, 1975; Griffin, 1978; Willaert *et al.*, 1978; Jone Kheere, 1981; Rivera *et al.*, 1981).

### Differentiation studies on free living amoebae

Band (1963) and Griffiths and Hughes (1968) developed replacement media to study the influence of diverse factors on differentiation. With this as the starting point and using *Acanthamoeba culbertsoni* (Singh and Das, 1970) as the test organism, extensive studies have been conducted in the Division of Biochemistry, Central Drug Research Institute,

Lucknow in the period 1969–1983 leading to the doctoral dissertations of Raizada (1972); Verma (1975), Kaushal (1976) and Srivastava (1982). Extension of the studies based on free living amoeba to axenically grown *Entamoeba histolytica* was the subject matter of the doctoral dissertation of Mitra (1975). The highlights of these investigations are summarised in the following sections.

### Encystation in non-nutrient medium

More than 80% of trophozoites of *Acanthamoeba culbertsoni* were transformed into cysts when they were placed in non-nutrient agar containing 80 mM sodium chloride, 15 mM magnesium chloride and 20 mM taurine. Encystation was inhibited by actinomycin D and cycloheximide. The following morphological changes are invariably noticed when trophozoites undergo encystation under the above conditions: reduced motility, gradual withdrawal of pseudopodia, rounding up, shrinkage in cell size and the final formation of an immotile double walled cyst (Raizada and Krishna Murti, 1971a,b; Raizada, 1972). When cysts formed in the minimal medium are transferred to a growth medium, the cysts undergo dormancy break and release motile and vegetative trophozoites.

Encystation of *A. culbertsoni* can also be induced by incubating trophozoites in the minimal medium incorporating epinephrine, norepinephrine, tyramine or 5 hydroxy tryptamine (Verma, 1975; Verma *et al.*, 1974a; Verma and Krishna Murti, 1976a). It is not necessary that trophozoites have to be continuously in contact with taurine or biogenic amines to be 'turned on' for differentiation. Exposure to these agents for 6 h or less is all that is required for the trophozoites to become committed to differentiation.

### Role of cyclic AMP in encystation

The triggering action of biogenic amines or taurine is mimicked by dibutyryl cyclic 3':5' adenosine monophosphate (Raizada and Krishna Murti, 1972a). During the transformation of trophozoites into viable cysts there is evidence for the accumulation of cyclic AMP as shown in figure 2. Taurine or biogenic amines get bound to specific receptors on the membrane of *A. culbertsoni* and activate a membrane bound adenylate cyclase (Raizada and Krishna Murti 1972a, 1973). Achar and Weissman (1980) have confirmed that increased intracellular levels of cyclic AMP are attained when *Acanthamoeba* cells in late log phase are transferred to an encystation medium. Encystation induced by taurine or epinephrine as well as the activation of adenylate cyclase are inhibited by actinomycin D or cycloheximide suggesting that regulatory

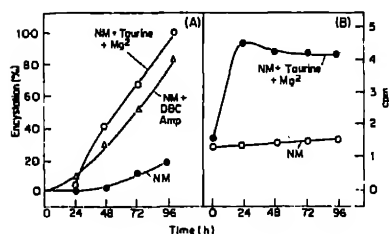


Figure 2. Differentiation of *A. culbertsoni* triggered by cAMP.



control of differentiation is exerted at the transcriptional and translational levels (Krishna Murti, 1975).

#### *Changes in chemical composition and biosynthesis*

Morphological changes observed during encystation of *A. culbertsoni* are accompanied by changes in the chemical composition and enzyme profile. The most dramatic change is the switching over of metabolism from aerobic to an anaerobic phase ending up eventually in total dormancy. Residual food materials such as glycogen and lipids are metabolized accompanied by a rapid turnover of phosphorylated compounds. During encystation there is a net loss of cellular DNA, RNA and protein but a concurrent gain in mucopolysaccharides and cellulose. However, incorporation studies with uracil-2- $^{14}\text{C}$ - and valine-1- $^{14}\text{C}$ - or leucine-1- $^{14}\text{C}$ - indicate synthesis of new species of RNA and protein. The most interesting change noticed is the high rate of incorporation of glucose-U- $^{14}\text{C}$ - into a polysaccharide which has been identified as cellulose by hydrolysis with fungal cellulase and recovery of labelled glucose from the hydrolysates. This is the first such report of the ability of a protozoan organism to synthesize cellulose which has been considered hitherto exclusive to the structure of plant cells. There was also rapid incorporation of glucosamine-1- $^{14}\text{C}$ - into mucopolysaccharides (Raizada and Krishna Murti, 1972b). Preliminary evidence for the acquisition of new serological characters has also been adduced in cells undergoing differentiation into cysts (Raizada *et al.*, 1972). Substantial decreases in the contents of RNA, triacyl-glycerides, glycogen and protein have also been recorded in *Acanthamoeba* during encystation (Bowers and Korn, 1969; Neff and Neff, 1969; Rudick and Weisman, 1973; Stevens and Pacher, 1973). Soluble proteins, amino acids, nucleotide derivatives and carbohydrates appear in the medium shortly after the onset of encystation (Neff *et al.*, 1964; Neff and Neff, 1969).

#### *Cyclic AMP phosphodiesterase*

Trophozoites of *A. culbertsoni* possess cyclic AMP phosphodiesterase activity (Raizada and Krishna Murti 1973). Imidazole, a known activator of cyclic AMP phosphodiesterase, did not inhibit the multiplication of *A. culbertsoni* in the axenic medium but prevented the epinephrine or taurine induced encystation of the trophozoites in the non-nutrient medium (Verma and Krishna Murti, 1975). Theophylline, a known inhibitor of cyclic AMP phosphodiesterase, could on the other hand induce encystation presumably by inhibiting the degradation of cyclic AMP. If one assumes that accumulation of cyclic AMP to an optimum 'critical' level is necessary for triggering the differentiation process, imidazole can be said to inhibit encystation by interfering with the accumulation of cyclic AMP. In contrast theophylline activates encystation by facilitating the accumulation of cyclic AMP.

#### *Turnover of cyclic AMP*

The intracellular concentration of cyclic AMP at any phase of cellular life reflects the turnover of this regulatory molecule involving the balance of synthesis by adenylate cyclase and degradation by cyclic AMP phosphodiesterase (Robison *et al.*, 1971). The activity of cyclic AMP phosphodiesterase of *A. culbertsoni* exposed to taurine or

epinephrine during starvation was significantly less than that of control cells incubating in the non nutrient medium. Some enzymic activity could be detected in the non nutrient medium whether the cells were exposed or not to taurine or epinephrine. The decay of phosphodiesterase is faster when cells are committed to differentiation triggered by epinephrine or taurine (Kaushal *et al.*, 1976). In contrast the adenylate cyclase activity is stimulated three fold under similar conditions (Raizada and Krishna Murti, 1972). Thus the stimulated adenylate cyclase and the fast decaying cyclic AMP phosphodiesterase together contribute to the build-up of relatively high concentrations of cyclic AMP required for controlling the biochemical events mediating the formation of macromolecules needed for structuring the cyst wall (Krishna Murti, 1973).

The nature of interaction between taurine or epinephrine and particulate subcellular fractions prepared from *A. culbertsoni* has been elucidated (Raizada and Krishna Murti, 1971, 1973; Verma and Krishna Murti, 1976a,b). Receptors for the binding of taurine- $[^{35}\text{S}]$ - and epinephrine- $[^{14}\text{C}]$ - were found to be located on the lipoprotein membranes. Subcellular particulate fractions prepared from trophozoites exposed to epinephrine prior to cell rupture exhibited a significantly higher adenylate cyclase activity than similar preparations made from cells unexposed to epinephrine. Binding of epinephrine to membraneous fractions of *A. culbertsoni* was inhibited by the drug propranolol, a well known  $\beta$ -adrenergic blocker. Interestingly enough, the same drug interfered with the induction of encystation of trophozoites by epinephrine. It may be noted that this is the first reported presence of  $\beta$ -receptors for catecholamines in amoebae.

#### *Catabolite repression*

Glucose inhibits encystation induced by taurine or epinephrine (Verma and Raizada, 1975). The glucose arising out of glycogenolysis during differentiation is channelled towards the synthesis of cellulose and glycoproteins. It is likely that the glucose added from outside is exerting a catabolic repression of the induction of enzymes needed for the biosynthesis of cellulose and glycoproteins. Catabolic repression is also exerted by acetic and citric acid. Catabolic repression induced by different nitrogenous compounds indicate that both nitrogen and carbon catabolite repression is involved in encystation of *A. culbertsoni* (Srivastava and Shukla, 1983).

#### *Polyamines and chemistry of effectors*

The demonstration of a protein which binds cyclic AMP in the cytosol fraction of *A. culbertsoni* is the final event in the story of encystation (Verma and Krishna Murti, 1976b). The nature of this protein remains to be elucidated. A direct and positive role of polyamines in the induction of encystation has been ruled out (Srivastava and Shukla, 1982). The ability of a number of structurally unrelated chemicals to bring about encystation suggests that an ideal encystation agent does not possess any specific molecular requirement. Aliphatic or aromatic nature or hydrophilicity or hydrophobicity do not appear to be the determinant characteristics. Higher concentration of magnesium may itself alter the membrane characteristics of amoeba resulting in encystation suggesting that even the presence of organic effectors is not absolutely essential. The diverse agents shown to induce encystation do however interact with

specific receptors on cell surface, induce conformational alterations and activate adenylate cyclase (Srivastava and Shukla, 1983a,b).

### Excystation

Excystation represents the reverse of what all we have discussed hitherto in relation to encystation. Kaushal (1976) and Kaushal and Shukla (1975, 1976, 1977a,b,c, 1978a,b,c) have standardized the conditions of growth of *A. culbertsoni* in a simple axenic medium the encystation of the trophozoites and the subsequent excystation of the cysts to viable trophozoites. The nature of the excystation effectors has also been elucidated.

#### *Morphological changes*

Gross changes noticed when a cyst hatches are shrinking of cytoplasm from the wall, movement of the amoeba within the wall occupation of one area of the inner wall by pseudopoidal movement, appearance of an opening in the wall, emergence of the amoeba from the cyst cast and in some instances total dissolution of the cast. (McConnachi, 1969; Singh and Das, 1970). The stages have been classified as maturation, activation, pre-emergent phase and eventual emergence (Matter and Byers, 1971). Scanning electron microscopy reveals the presence of ostioles on the surface and the formation of holes (Chamber and Thompson, 1972).

#### *Excystation factors*

Axenically prepared cysts of *A. culbertsoni* readily excysted in the presence of heat-stable factors present in aqueous extracts of *Escherichia coli*, *Klebsiella aerogenes*, *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus subtilis*, *Bacillus megaterium*, *Aspergillus niger*, *Chaetomium globosum*, *Myrothecium verrucaria*, *Trichoderma viridi*. Peptones and protein hydrolysates also induce excystation. No linear relationship is observed between the amount of the agent and the degree of excystation. Continuous presence of excystation factor is essential and the removal of the agent stops further excystation. Presumably no triggering mechanism is involved (Kaushal and Shukla, 1977a) in contrast to encystation where activation of adenylate cyclase is a prerequisite.

Detailed fractionation studies using aqueous extracts of *E. coli* indicate that the effectors of excystation are heat stable, dialysable and comprise low molecular weight substances. Most of the excystation promoting activity is associated with a fraction rich in amino acids. Glutamic acid shows the highest activity followed by serine, threonine and alamine (Kaushal and Shukla, 1977a,b).  $\gamma$ -Amino butyric acid with an amino and carboxyl group and an intervening flexible chain of 3-carbon atom appears to have the optimum structural requirement for inducing excystation. These features are present in glutamic acid. Compounds which possess one or the other feature of these basic requirements promote varying degrees of excystation (Kaushal and Shukla, 1977c).

#### *Mechanism of excystation*

Mitomycin C and Actinomycin D have practically no effect on excystation whereas cycloheximide exerts a 100% inhibition. Dinitrophenol and sodium arsenite also block

encystation induced by glutamic acid. The data presented in figure 3 reveal that there is significant increase of protein, marginal increase of RNA but very little DNA synthesis during excystation. There is negligible incorporation of thymidine- $^{3}\text{H}$ - into DNA but a rapid incorporation of uracil-2- $^{14}\text{C}$ - into RNA and incorporation of chlorella protein hydrolysate-U- $^{14}\text{C}$ - into proteins take place. Studies with nucleic acid and protein synthetic inhibitors suggest the involvement of the process of translation but not transcription in excystation (Kaushal and Shukla, 1978a,b).

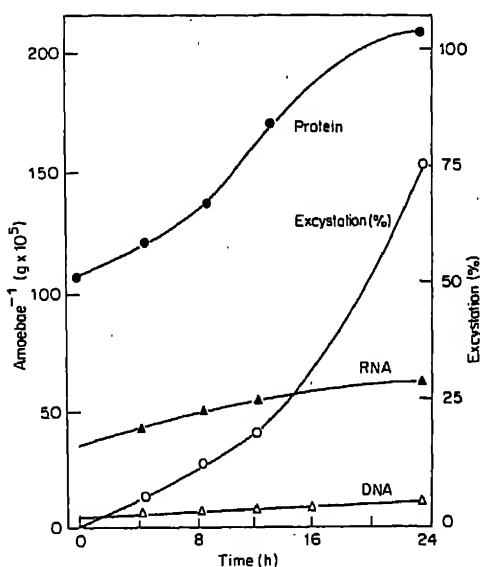


Figure 3. Macromolecular content and excystation of *A. culbertsoni*.

#### Release of depolymerizing enzymes

Autoclaved cysts of *A. culbertsoni* provide carbon sources for the growth of an *Aeternaria* sp. The fungus degrades the cysts, secretes into the medium protease, cellulase and chitinase and digests the cysts (Verma *et al.*, 1974). Excystation of *A. culbertsoni* in a liquid medium containing peptone and sodium chloride or aqueous extracts of *E. coli* is accompanied by secretion of two proteases, cellulase and chitinase (table 2). The proteases are secreted during the early phase whereas the disappearance of empty cysts or casts begins 48 h later coinciding with the release of cellulase and chitinase. Formation of an opening on the cyst wall is an essential step in excystation and Neal (1960) has suggested that the escape hole is produced by the action of enzymes located or synthesized in the cell membrane. The early secretion of proteases by cysts of *A. culbertsoni* suggests that they may have an important role to play in excystation. Cellulase and chitinase, on the other hand, are secreted only after excystation is completed and may be important in the subsequent degradation of empty cyst walls or the casts (Kaushal and Shukla, 1976). The alkaline protease released during excystation has been purified 100 fold by Sephadex G-100 chromatography and shown to have a molecular weight of 21400, pH optimum of 8-9 and a temperature optimum around

**Table 2.** Excystation of *A. culbertsoni* in presence of aqueous extract of *E. coli* and release of enzymes.

	24 h	48 h	72 h	96 h
Excystation (%)	66	73	74	74
Degradation of empty cysts (%)	Nil	45	81	100
Cellulase activity in medium	Nil	Nil	307	405
Chitinase activity in medium	Nil	Nil	420	970
Protease I (pH 5) activity in medium	450	1500	1040	Nil
Protease II (pH 9.5) in medium	735	4095	5120	3720
Cellulase activity	$\mu\text{g}$ glucose liberated in 60 min by 100 ml			
Chitinase activity	$\mu\text{g}$ N-acetyl glucosamine 60 min by 100 ml			
Protease I and II activity	$\mu\text{g}$ tyrosine liberated in 60 min by 100 ml			

From Kaushal and Shukla, (1976).

55°C. The enzyme is inhibited by phenyl methyl-sulphonyl fluoride and appears to be a serine protease (Kaushal and Shukla, 1978c). In an extended study using cellulose labelled with glucose-U-[ $^{14}\text{C}$ ]- as substrate, cellulase activity could be detected in the early phase of excystation. Dormant cysts do not possess protease, cellulase or chitinase activities. Protease I and protease II appear in the excysting cell in 24 h, reach the maxima on the 2nd day and decline thereafter. Cellulase and chitinase also behave in a similar manner. In the culture medium the appearance of enzymes shows a slightly different pattern suggesting that all the depolymerizing enzymes are associated with the cells or are synthesized and then only partially released. Incubation of the cysts with a mixture of the three depolymerizing enzymes does not bring about excystation but causes many morphological alterations (Kaushal and Shukla, 1978a,b).

### Differentiation of parasitic amoebae

Information on the morphology, nutrition and general biochemistry of *Entamoeba histolytica* is far from adequate to be of any critical use in the design of drugs or other therapeutic measures (Mitra, 1975). *E. histolytica* can be maintained in the laboratory along with associate intestinal microflora. Virulent cultures have been used to simulate human amoebiasis in experimental animals. Intracaecal inoculation of such cultures into young rats or cats or into the liver of hamsters have been employed to give models for intestinal amoebiasis and amoebic hepatitis respectively. The introduction of the technique of axenization by Diamond (1968a,b) has also been not of much help in achieving infection by bacteria-free amoebae or in producing cysts.

Cyst formation can be readily demonstrated in *E. histolytica* cultures maintained with associate bacteria (Balamuth, 1951; Balamuth and Weiboldt, 1951; Dobell and Neal, 1952; Diamond, 1968a,b). However, the results obtained with axenic cultures have not been unequivocal. One of the few leads we have today in a positive direction in this regard is the outcome of the painstaking work of Mitra (1975) who devised a technique of prior exposure of axenic cultures to cholera toxin in order to prime the triggering action of epinephrine. Axenically grown *E. histolytica* does not bind epinephrine unless it is exposed to cholera toxin. Population of amoebae exposed to a combination of

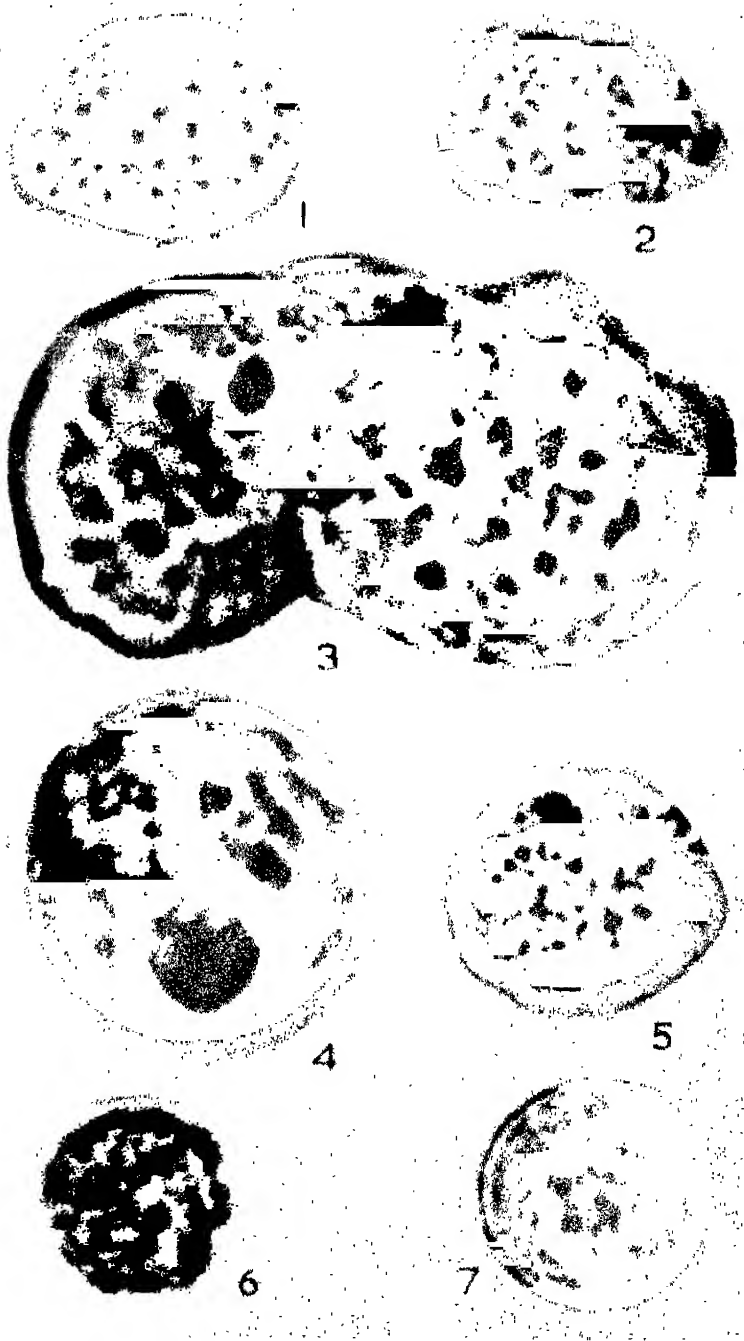


Figure 4. Morphological changes in axenically grown *E. histolytica* exposed to toxin and epinephrine.

treatment schedules which include the allowing of amoebae to ingest starch particles and exposure to cholera toxin followed by bathing in epinephrine exhibit a variety of interesting morphological changes (figure 4). It has not been possible so far to ascertain whether the round bodies thus formed are the polynucleated mature cysts passed by human carriers and which on ingestion by susceptible hosts excyst in the colon and multiply to give rise to amoebic dysentery (Mitra and Krishna Murti, 1978).

The questions that remain to be answered in regard to *E. histolytica* are:

- (i) Is there a loss of surface receptors of biogenic amines or other encystation effectors when *E. histolytica* trophozoites are axenized?
- (ii) What are the factors present in the milieu of the human gut and its commensal microflora which could be acting as encystation and excystation effectors?
- (iii) Does the loss of virulence and invasiveness associated with axenization also mean the loss of the ability of the cells to receive the chemical signals of encystation effectors?

### Molecular biology of differentiation of amoeba

The cascade of events occurring in the differentiation of *A. culbertsoni* is summarised in figure 5. The inhibitory effect of Actinomycin D on encystation under different conditions suggests that the control is exerted at the transcriptional level. Catabolic repression by glucose, acids and in general by carbon and nitrogen could be taken as suggestive evidence of the blocking of transcription by repressors synthesized as the

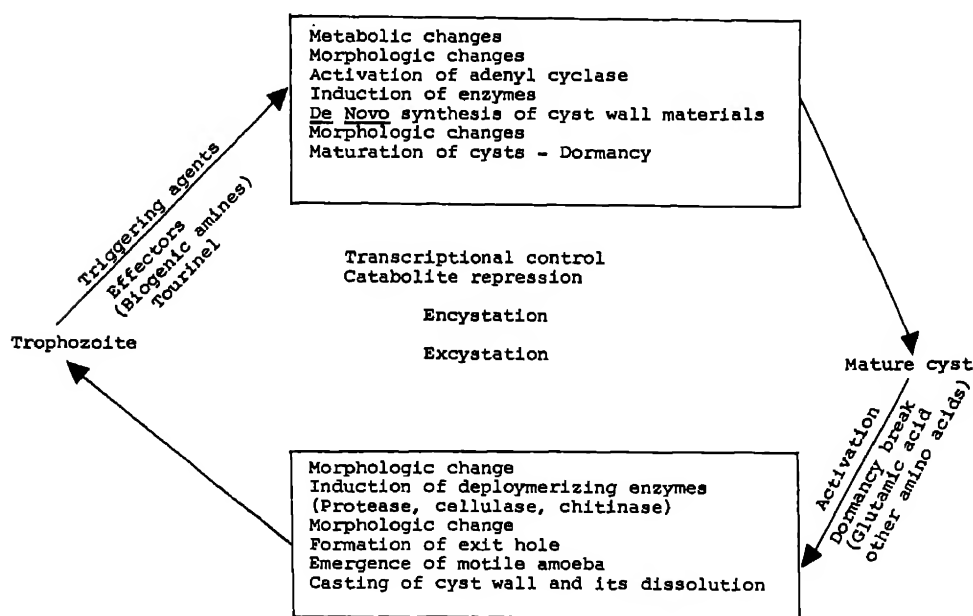


Figure 5. Cascade of events in differentiation of *A. culbertsoni*.

trophozoites age. The inhibition imposed is unmasked by the effectors which trigger encystation through the intermediately messenger cyclic AMP. Phosphorylation of cytosol proteins can be assumed to occur as a logical sequel leading to conformational alterations and consequently to new functional activity. Reserve food materials like glycogen and lipids are hydrolysed. Glucose is used directly for building cellulose and after amination changed to glycoproteins. The enzymes required for the biosynthesis of the building materials are induced by derepression. Polyamines known to regulate derepression in many prokaryote and eukaryote cells do not seem to be involved in the differentiation of *A. culbertsoni*. Morphological changes facilitate the withdrawal of pseudopodia and the layering of the outer walls and their maturation into the impermeable coat.

The excystation of cysts bears a gross resemblance to the germination of bacterial spores or vegetable seeds in the matter of the requirement of an activation of "awakening" phase when the dormant amoeba is activated from its sluggish state to one of rapid movement. Glutamic acid and other amino acids function as effectors for this activation by an as yet unelucidated mechanism. Activation and dormancy break are inhibited by cycloheximide suggesting that the control is exerted at the translational level. Depolymerizing enzymes are induced. The proteases attack peptide bonds on the structural constituents causing the appearance of exit passages or escape holes. Cellulase and chitinase also help in this process but much more in solubilizing the case or the empty cyst wall and releasing nutrients for the growth of amoebae.

Divalent cations, particularly, magnesium and possibly calcium appear to be essential for the biochemical reactions in both encystation and excystation and the fluxes of these ions help presumably in maintaining osmotic balance and in the attainment of the final architecture of the cysts in encystation as well as in the loosening of the cyst wall during excystation.

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## Liposomes in immunology

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**Abstract.** Liposomes, the artificial phospholipid vesicles, have the capacity of entrapping water soluble substances in their aqueous compartments. Of the many possible potentials of liposomes their application in immunology is most significant. Recent studies have shown an adjuvant and a carrier effect of liposomes to a number of antigens. Liposomes used in these studies are generally multilamellar vesicles with the antigen encapsulated in the aqueous phase. Some antigens may also be associated with the lipid lamellae covalently or noncovalently. The adjuvant property of liposomes is greatly affected by the surface charge of the vesicle as well as the site of association of the antigen. The other factors which may have a role in immunopotentiality by liposomes are the size and structure of the vesicles, the lipid composition, route of administration and their surface sugars. In addition, liposomes may function as carriers to haptens and other antigens. In association with liposomes the nature of the immune response may be modulated. For a further enhancement of the adjuvant activity of liposomes use has been made of immunomodulators.

**Keywords.** Liposomes; adjuvant; protein antigens.

### Introduction

Liposomes are artificial vesicles comprised of lipid and aqueous compartments where the lipid exists in the bilayer form. Such vesicles can be composed solely of phospholipids or in combination with other amphipathic molecules such as sterols, long chain organic bases or acids. When phospholipids are suspended in an excess of aqueous solution they spontaneously form multilamellar concentric bilayers with lipid layers separated by layers of aqueous medium. Water soluble substances such as drugs, proteins, nucleic acids and dyes, present in the aqueous phase during the formation of liposomes, can be encapsulated into the aqueous compartments of the vesicles. This unique property of liposomes has made them a versatile tool for an increasing number of studies in biology and medicine.

The earliest suggestion of a therapeutic potential for liposomes was for its possible application as carriers of enzymes and drugs *in vivo* in the therapy of various metabolic and physiological disorders (Gregoriadis *et al.*, 1971). The concept behind the use of

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Abbreviations used: CFA, Complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; MLV, multilamellar vesicle;  $T_c$ , temperature; SPDP, succinimyl  $\beta$ -(2-pyridyldithiopropionate); BSA, bovine serum albumin; GCSA, gross cell surface antigen; ULV, unilamellar vesicles; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearyl phosphatidylcholine; MDP, muramyl dipeptide; MAF, macrophage-activating factor.

liposomes as carriers of drugs and macromolecules was mainly related to an expected protection of the encapsulated molecules in the blood stream. On the contrary, it was found that liposome encapsulation of antigens resulted in elevated antibody titres in comparison to the free antigen (Allison and Gregoriadis, 1974). This interesting observation opened the field for the study of the immunopotentiating properties of liposomes. The common adjuvants used for experimental immunization in laboratory animals are Freund's complete and incomplete adjuvants (CFA and IFA). They are composed of a water-in-oil emulsion with or without heat killed mycobacteria. Though these adjuvants evoke high level and long lasting immunity they are not suitable for use in humans since they cause the formation of granulomas at the site of injection. Other adjuvants such as alum, aluminium hydroxide and calcium chloride used in humans are far from ideal. Hence, a need is felt for a safe and effective adjuvant for use in human immunization. Liposomes have a decided advantage over the above mentioned adjuvants since they are prepared from biodegradable phospholipids which do not produce granulomas (Allison and Gregoriadis, 1976). Further, liposomes protect the entrapped antigens from the hypersensitivity reactions (Gregoriadis and Allison, 1974). The adjuvant and carrier property of liposomes to a number of antigens has been established in recent years (van Rooijen and van Nieuwmege, 1981; Alving and Richards, 1983). Considering the potential of liposomes in immunopotentialiation it is likely that their application in this field may be realized first.

In addition to the development of liposomes as immunopotentiators, the vesicles have been used earlier in investigating a wide range of immunological events. These include lysis of foreign cells as a consequence of membrane damage and the ability of membrane sensitized haptenic antigens to induce formation of antibodies and/or cytotoxic effector cells. In the present review attention will be directed to the adjuvant properties of liposomes with respect to protein antigens and as carriers of haptens.

### Preparation of liposomes

Liposomes generally used in the immunological studies are multilamellar in structure. The ease of encapsulation of proteins and haptenic groups and the variety of lipid compositions used in the preparation of multilamellar vesicles (MLVs) makes them the choice for these studies. The method described by Bangham *et al.* (1974) for the preparation of MLVs is exceptionally simple. The lipid mixture taken in organic solvents is allowed to deposit upon the walls of a round bottomed flask by rotary evaporation under reduced pressure. The thin film of lipids is dispersed in aqueous buffer at a temperature above the transition temperature ( $T_c$ ) of the lipid or above the  $T_c$  of the highest melting component in the mixture.

A higher percentage of encapsulated aqueous phase per mol of lipid may be achieved by a long hydration and gentle shaking than a faster and vigorous preparation. The entrapped volume of the MLVs can be further increased by including charged lipids in the bilayer. The main disadvantages of MLVs are the heterogenous population of vesicles and relatively low ratio of internal aqueous space per total lipid. A more uniform preparation can be obtained by passing the vesicles through polycarbonate membrane (Olson *et al.*, 1979) or by extrusion through a French Press (Barenholz *et al.*, 1979).

## Association of materials with liposomes

The original and still most commonly used mode of association of materials with liposomes is the aqueous phase entrapment of water soluble molecules. However, macromolecules such as proteins during entrapment in the aqueous phase may bind to the surface of liposomes (Tyrrell *et al.*, 1976). This is similar to membrane-protein interaction which may be electrostatic or hydrophobic in nature. Such a preparation results in antigens exposed on the surface of liposomes. For a specific surface expression of antigens, preformed liposomes are incubated with the protein (Raphael and Tom, 1984). For an incorporation of the materials in the lipid bilayers the nonpolar molecules are added to and dried down with the lipid phase in organic solvent (Bangham *et al.*, 1974). The higher the solubility of the molecules in the non-polar solvent the greater the amount which can be sequestered in the hydrophobic regions of the liposomes. A more stable surface expression of antigens is obtained by covalent coupling of these molecules to preformed liposomes. Four general methods have been described in literature for the coupling of proteins to the surface of liposomes.

*Periodate oxidation of vicinal cis-hydroxyl groups of oligosaccharide chains of glycoproteins followed by reduction of Schiff's bases formed between the amino groups of the phospholipids of the liposomes*

For example, horse raddish peroxidase has been covalently coupled to preformed liposomes containing phosphatidyl ethanolamine, stearylamine or phosphatidyl serine (Heath *et al.*, 1980). The major disadvantage of this method is that it can be applied for coupling of only those proteins having available oligosaccharide residues for the generation of aldehydes. However, in recent years a number of novel methods have been developed for glycosylation of proteins. This method may then be applied for the glycosylated proteins.

*Periodate oxidation of the oligosaccharide chains or glycosphingolipids of liposomes followed by reduction of the Schiff's bases formed between the amino groups of the proteins and aldehyde groups of the lipid*

In this way proteins such as immunoglobulins and  $F(ab')_2$  fragments have been covalently coupled to vesicles (Heath *et al.*, 1981). This method, too, has a disadvantage as it necessitates the presence of glycosphingolipids in the liposome membrane.

*Homobifunctional cross linking between the amino groups of phospholipids and proteins mediated by glutaraldehyde, dimethyl suberimidate or carbodiimide*

Torchilin *et al.* (1978) used these coupling agents and a stable immobilization of  $\alpha$ -chymotrypsin on the surface of liposomes was achieved. However, utilization of homobifunctional coupling agents might lead to the formation of homopolymers of vesicles, protein or both.

*Chemical cross linking using heterobifunctional agents*

In order to minimize homopolymerization and intramolecular cross linking the use of heterobifunctional agents has been suggested. An example of such a reagent is N-

hydroxyl-succinimyl  $\beta$ -(2-pyridyldithiopropionate) (SPDP) for covalent coupling of antibody and *S. aureus* protein A to liposomes (Laserman *et al.*, 1980). For coupling of protein to liposomes SPDP is first reacted with phosphatidylethanolamine and the stable derivative PE-DTP is used for the formation of the liposomes. Subsequently, proteins containing activated thiol groups are coupled to the liposomes. A similar method, introduced by Martin and Paphadjopoulos (1982) involves the formation of a phospholipid derivative N-[4-(*p*-maleimidophenyl) butyryl] phosphatidyl ethanolamine. The maleimide moiety incorporated in liposomes is reacted with the sulphhydryl groups of *F(ab')* fragments leading to an efficient and stable protein-vesicle linkage. Recently a modified method involving thiolation of antigen and coupling to maleimide moiety of preformed liposomes has been described for proteins lacking sulphhydryl groups (Shek and Heath, 1983).

Haptens such as lipids and peptides are incorporated in liposomes by covalently coupling them to phosphatidylethanolamine prior to liposome preparation. This method of liposome sensitization has been found to enhance the immuno-genicity of a wide variety of agents (Kinsky, 1980; van Houte *et al.*, 1981).

### Adjuvant Properties of liposomes

The adjuvant effect of liposomes was first reported by Allison and Gregoriadis (1974) for the antigen, diphtheria toxoid. Since then the immunopotentiating effect of liposomes has been the subject of an increasing number of studies. The immunogenicity of a wider variety of antigens for example proteins (van Rooijen and van Nieuwmegen, 1980b; Heath *et al.*, 1976), peptides (Liftshitz *et al.*, 1981), sugars (Das *et al.*, 1982a,b) and lipids (Alving, 1977) have been found to be significantly enhanced in association with liposomes.

The properties of the liposomes may be varied at will. For instance the vesicle size phospholipid composition, surface charge and lamellar structure may be altered as desired. As a consequence, the immunopotentiating capacity of the vesicle to the associated antigen also changes. A number of parameters affecting the adjuvant property of liposomes have been studied in the past decade which is discussed below.

### The effect of surface charges of the vesicles

Liposomes may be neutral, negative or positively charged depending on the lipid used in the liposome preparation. The effect of surface charge on the immunopotentiating activity of liposomes was first observed by Allison and Gregoriadis (1974). It was found that inoculation of diphtheria toxoid in negatively charged liposomes elicited significantly higher antibody levels than when entrapped in neutral or positively charged liposomes. A later report showed that though negatively charged liposomes could act as adjuvants, positively charged liposomes too could do the same (Heath *et al.*, 1976). Similar results were obtained by van Rooijen and van Nieuwmegen (1980b) showing that positively charged and neutral liposomes have the same adjuvant activity as negatively charged liposomes. Still most of the workers studying the adjuvant properties of liposomes to antigens made use of negative liposomes. Recently the

effectiveness of positively charged liposomes in producing significant levels of antibodies has been demonstrated (Latif and Bachhawat, 1984a). The antibody titres obtained with lysozyme entrapped in positively charged liposomes has been found to be higher than neutral, negatively charged liposomes and even CFA. The high level of immunopotentiality by positive liposomes cannot be attributed to a higher affinity of the antigen for these liposomes since both carry the same charges. In contrast to previous reports that liposomes do not cause granuloma formation (Allison and Gregoriadis, 1976) it was found that positively charged liposomes, with or without antigen, lead to the formation of mild granulomas at the sites of injection. Though the exact mechanism of action of the positively charged liposomes is not understood, it is clear that these liposomes interact differently with cells *in vivo* in comparison to the neutral and negatively charged liposomes possibly eliciting a cell-mediated immunity, in addition. There is a possibility that the positive charge may hamper the fusion of the vesicles with the lysosomes thus allowing protection and prolonged exposure of the entrapped antigen to the immune system. Further studies on the interaction of liposomes with cells in culture may provide an insight into the mode of action of these vesicles. For the present, the profound immunopotentiating activity of positively charged liposomes is of considerable interest.

Table 1. Antibody response of rabbits to lysozyme

Immunogen*	Antibody titres in weeks**				
	1	2	3	4	5
	Primary response		Secondary response		
Lysozyme in saline†	ND	ND	4.67	ND	5.3
Lysozyme plus CFA†	ND	ND	213.3	ND	170.7
Lysozyme entrapped in neutral liposomes†	1	1.3	26.7	16	37.3
Lysozyme entrapped in negatively charged liposomes†	0.7	2	22.7	22.7	53.3
Lysozyme entrapped in positively charged liposomes†	2.3	85.3	384	426.7	768
Lysozyme coupled to neutral liposomes‡	4.3	17.3	37.3	26.7	64
Lysozyme coupled to neutral liposomes plus CFA‡	2	192	512	853.3	1706.7
Lysozyme entrapped in $\beta$ -Gal liposomes§	1.3	2	37.3	42.7	42.7
Lysozyme entrapped in $\alpha$ -Man liposomes§	1	1	3	4.7	12

\* Subcutaneous injections of lysozyme administered at 0, 2 and 4 weeks.

\*\* Sera assayed by passive haemagglutination and expressed as the reciprocal dilution of the end point. Each datum point represents the mean response of three animals.

† Latif and Bachhawat, 1984a.

§ Latif and Bachhawat, 1984b.

‡ Latif, 1984.

ND Not done.

### The effect of the sites of association of the antigen with the liposome vesicles

As discussed earlier there are a number of ways by which proteins can be associated with liposomes and this depends upon the physicochemical properties of the proteins.



Water-soluble molecules may be entrapped within the aqueous compartments between the lipid lamellae whereas hydrophobic proteins may interact with the lipid bilayers (Tyrrell *et al.*, 1976). Further, amphipathic substances may be associated with the lipid bilayers; at the same time their hydrophilic tails may project on the surface of the liposomes (Fendler, 1980). Recent interest has been centred on the effect of the modes of association of antigen with liposomes on antibody production. Entrapment of a variety of antigens in liposomes has been found to induce enhanced humoral responses compared to similar proteins injected free in saline (Allison and Gregoriadis, 1974; van Rooijen and van Nieuwmegen, 1977; Hudson *et al.*, 1979). That a liposome protein association was a prerequisite for the adjuvant effect was evident when mixtures of free antigen and liposomes did not lead to any immune stimulation (Shek and Sabiston, 1982b). During encapsulation in liposomes some antigens for example serum proteins, may associate with the liposome surface membrane (Hoekstra and Scherphof, 1979). Conflicting reports have appeared regarding the significance of surface exposure (van Rooijen and van Nieuwmegen, 1980a) versus internal entrapment (Six *et al.*, 1980) of the antigen in enhancing the immunogenicity of liposome-associated antigens.

Van Rooijen and van Nieuwmegen (1977, 1978) reported comparable anti-body titres elicited by human serum albumin and bovine  $\gamma$ -globulin adsorbed on and entrapped within the liposomal vesicles. Subsequently it was postulated that only those ligands that are exposed on the liposome surface are immunogenic. However, substantial data to prove or disprove this hypothesis was lacking. On the contrary internalizing of a number of antigens in liposomes was reported to induce antibody production (Allison and Gregoriadis, 1974; Hudson *et al.*, 1979). The significance of surface adsorbed and entrapped antigens in mediating immune responses has been further investigated using bovine serum albumin (BSA) and another serum protein (Shek and Sabiston, 1982b). It has been found that antigens exposed on the surface of liposomes are immunogenic. At the same time trypsinization of liposome encapsulated antigens does not reduce or abolish the immunological response. Additional information regarding the immunogenicity of entrapped antigen has been obtained using lysozyme as an antigen (Latif, 1984). Lysozyme does not associate with the liposome surface and as such it leads to almost negligible antibody production. However, when entrapped in liposomes the antibody titre is highly significant.

Viral antigens are mostly membrane proteins and when incorporated in liposomes may associate with the aqueous compartments as well as the lipid membranes. Such a polypeptide derived from Hepatitis B surface antigen showed a high percentage of incorporation in liposomes (Sanchez *et al.*, 1980). When administered in guinea pigs higher antibody titres were obtained with liposome-associated antigen than the antigen administered in aluminium gel. Similarly, gross cell surface antigen (GCSA), extracted from syngenic (C58NT)D lymphoma cells and incorporated in liposomes showed a strong association with the lipid membranes and only 25% of the total protein was trapped in the aqueous compartment. Immunization with liposome-associated GCSAa showed that the antigen present on the liposome surface could induce antibodies to GCSAa reaching in some instances the level obtained after immunization with viable syngenic tumour cells (Gerlier *et al.*, 1980). The immunogenicity of surface expressed antigens has been further demonstrated using human LS174T colon tumour cell membranes incorporated in vesicles (Raphael and Tom, 1984).

Proteins, as described earlier could be associated with the liposomes by covalent linkage with the vesicles. However, the effect of such an association on immunopotentiality by liposomes has not been extensively investigated. Recently this phenomenon for enhancing the immunogenicity of BSA has been employed by Shek and Heath (1983). BSA covalently linked to the surface of preformed unilamellar vesicle composed of phosphatidyl choline, cholesterol and N-(4-maleimidophenyl butyryl) phosphatidyl ethanolamine has been found to be immunogenic. A simpler method for coupling lysozyme to liposomes through phosphatidyl ethanolamine has been carried out earlier (Latif and Bachhawat, 1981). The surface-coupled antigen has been found to be significantly immunogenic. However, in association with another adjuvant, CFA coupled ligands are several fold more immunogenic than the native antigen in CFA (Latif, 1984). These studies demonstrate the potential of liposomes as carriers and adjuvants to protein antigens and may find application in vaccine preparation.

### Effect of size and structure of liposomes

The size and structure of liposomes may be modulated as required. It is anticipated that these factors may affect the immunogenicity of liposome associated antigens. However, comparative studies of the immunogenicity of antigens associated with different preparations of liposomes are not available. Most of the immunological studies on liposomes have been carried out using MLVs. Recently the differences in multilamellar and unilamellar liposomes of comparable size in promoting antibody response to a protein antigen has been analyzed (Shek *et al.*, 1983). Results obtained with the negatively charged liposomes prepared from dimyristoyl-lecithin indicate that unilamellar vesicles (ULVs) are more effective than MLVs in promoting an immune response to the entrapped BSA. Though the exact mechanism responsible for the difference in the immunopotentiating capacity of the two liposome preparations remains to be established, it is postulated that the extent of BSA molecules embedded in the phospholipid bilayers of the two kinds of vesicles might have a role to play.

### Effect of lipid composition

Most of the liposomal vesicles for immunological studies have been prepared from egg lecithin, cholesterol and a negatively charged phospholipid, diacetyl phosphate or phosphatidic acid. In order to introduce a positive charge stearylamine is used. The molar ratio of the lipids used in the preparation of liposomes which promotes effective antibody production (Allison and Gregoriadis, 1974) is usually 7:2:1 of egg lecithin, cholesterol and charged lipid, respectively. However, whether this ratio is indeed optimum for immune enhancement remains to be established.

Egg lecithin or phosphatidylcholine is the most important ingredient of liposomes used for adjuvant activity. It is bio-degradable and a harmless compound when administered as liposomes (van Rooijen and van Nieuwmegen, 1980b) although some exchange may occur with the phospholipid of cells (Gregoriadis *et al.*, 1977). The most important advantage of phosphatidylcholine liposomes as adjuvants is that, in contrast to phosphatidyl inositol, phosphatidyl glycerol and phosphatidic acid, phosphatidyl-

choline is a very poor antigen (Alving, 1977). Liposomes prepared with phosphatidylcholine by themselves, do not evoke an immune response in rabbits even when incorporated in IFA. However an immune response against phosphatidylcholine is induced when lipid A is incorporated in the liposomes (Shuster *et al.*, 1979).

Liposomes prepared from sphingomyelin have been reported to be more effective in eliciting an immune response to incorporated antigen than liposomes from phosphatidylcholine (Uemura *et al.*, 1974; Yasuda *et al.*, 1977). There is a striking difference in the  $T_c$  of these lipids (Phosphatidylcholine  $-8^\circ$  to  $15^\circ$ , sphingomyelin  $42^\circ$ ) and it is suggested that high immunological response by sphingomyelin liposomes may be due to their greater stability. In contrast to these observations van Rooijen and van Nieuwmegen (1980b) reported lower adjuvant activity of sphingomyelin liposomes than phosphatidylcholine liposomes to the associated protein antigen.

Liposomes composed of dipalmitoyl phosphatidyl-choline (DPPC) and distearyl phosphatidylcholine (DSPC) ( $T_c$   $41.4^\circ$  and  $54.9^\circ$ , respectively) have also been reported to be more effective immunogens than those prepared from egg lecithin (Hudson, 1977). It is postulated that these liposomes may have greater bilayer stability at physiological temperature and may thus persist longer *in vivo* than egg lecithin. In contrast, it has been found that though DPPC and DSPC liposomes are strong immunopotentiators to the entrapped antigens liposomes prepared from egg lecithin are better adjuvants (Latif and Bachhawat, 1984a).

### Effect of the route of administration

For potentiating an immunological response to liposome-associated antigens, animals may be immunized intravenously, intraperitoneally, intramuscularly or subcutaneously. A strong primary immune response is induced by intravenous administration of liposome-associated antigens (Allison and Gregoriadis, 1974; Heath *et al.*, 1981). However, the response does not persist and may not show a secondary induction. In contrast, intramuscular and subcutaneous route elicit higher concentration of antibodies (Allison and Gregoriadis, 1974; Heath *et al.*, 1976) on secondary immunization. Intraperitoneal injections of liposome-associated antigens too, induce a substantial antibody production (Shek and Sabiston, 1982a,b).

After intravenous injection liposomes are removed from the circulation by reticuloendothelial cells and are rapidly degraded (Tyrrell *et al.*, 1976). Thus accumulation of liposomes in order to trigger a high antibody response does not take place. Still, significantly higher titres of antibody were obtained after intravenous administration of liposome entrapped antigen than the free foreign protein (Allison and Gregoriadis, 1974; Hudson *et al.*, 1979). The adjuvant property of intravenously administered liposomes is evident from the observation that liposome carrier itself could activate phagocytic cells in the reticuloendothelial system (Hudson *et al.*, 1979). Significant stimulation of the immune system is observed when liposomes are injected subcutaneously intramuscularly or intraperitoneally. It is possible that liposomes injected by these routes will remain at the site of injection for a long period. Thus liposomes exert their adjuvant effect by retaining a 'depot' of an antigen at the site of injection (Tyrrell *et al.*, 1976). However, the adjuvant action is not entirely physical.

Some interaction between liposome and cells of the immune system has been reported (Hudson, 1977). The appearance of activated mononuclear phagocytes and increased specific activities of several lysosomal hydrolases in these cells by a single liposomal sensitization in mice (Hudson *et al.*, 1979) indicates that liposomes may affect the reticuloendothelial function directly by activating phagocytic cells.

### **The effect of sugars on the surface of liposomes**

Most adjuvants exert their major effects in macrophages (Allison, 1979). However, the mechanism of cellular action of liposomes in stimulating antibody production remains to be established. It is clear that liposomes administered either intravenously, intraperitoneally or subcutaneously are sequestered by the organs of high reticuloendothelial activity such as liver, spleen, lymph nodes (Tyrrell *et al.*, 1976). Thus they finally gain access to the phagocytic cells of the reticuloendothelial system which are responsible for the clearance of the liposomes. However, the participation of these cells *i.e.*, the macrophages, in potentiating the immune response to liposome-associated antigens remains unresolved. Recently it has been shown that macrophages are necessary for the induction of a humoral response to liposome-associated protein antigens (Shek and Lukovich, 1982). Liposomes designed specially to interact with macrophages would facilitate a better immunopotential. It is well established that macrophages possess recognition sites for sugars such as mannose, galactose and fucose (Weir, 1980). Liposomes carrying terminal galactose and mannose residues are specifically recognized by lecithin-like molecules present on the plasma membranes of macrophages of parenchymal and nonparenchymal tissues (Ghosh and Bachhawat, 1980). A recent study of the immunological response of sugar grafted liposomes shows that antigen entrapped liposomes bearing galactose on the surface induce an immune response comparable to sugar-free neutral liposomes. However, the immune response by mannose-coupled liposomes is almost equal to that of the free antigen (Latif and Bachhawat, 1984b). Based on these results, it is postulated that subcutaneous administration of liposomes facilitates a receptor mediated uptake by peritoneal macrophages and macrophages derived from bone marrow by a recognition system specific for mannose. A greater accessibility of these liposomes to the phagocytic cells leads to a rapid degradation of the antigen and subsequently low immune response results.

### **Liposomes as carriers of antigens**

In addition to their adjuvant effect, liposomes have been recognised as efficient carriers of antigens in recent years. Haptenic groups are generally inserted in liposomes by coupling them to one of the phospholipids used in the liposome preparation. With such liposomes, Kinsky (1978) investigated the immune response of haptens such as dinitrophenyl and phosphorylcholine. The most obvious advantages of the use of liposomes as carriers are the easy biodegradability and the low immunogenicity of liposomes compared to protein carriers thus eliminating the need to remove carrier

specific antibodies. These potentials of liposomes attracted extensive research in the production of antibodies to a number of haptens.

This approach for obtaining specific antibodies has been extended to molecules of clinical interest such as hormones, drugs, vitamins etc. For example, anti-L-thyroxine antiserum has been produced with liposomes sensitized with L-thyroxine derivative of phosphatidylethanolamine (Tan *et al.*, 1981). In addition, extensive studies on the immunogenic properties of haptened liposomes have been carried out using tripeptide enlarged haptens such as 3-(*p*-azobenzene arsonate)-N-acetyl-L-tyrosylglycylglycine and N-(2,4-dinitrophenyl)- $\beta$ -alanylglycylglycine (van Houte *et al.*, 1981). Wood and Kabat (1981) showed the production of antiglycolipid antibodies and Das *et al.* (1982a,b, 1984) report the production of antibodies against sugars such as mannose, galactose and N-acetyl-D-glucosamine using liposomes as carriers. The carrier effect of liposomes to protein antigens has also been reported (Latif, 1984).

### Nature of the immune response mediated by liposomal antigens

For a better understanding of the mechanism of adjuvant action of liposomes to associated antigens and for their applicability in vaccines, information regarding the kind of immunoglobulin produced by liposomal antigen is required. Protein antigens are generally T-dependent with respect to eliciting antibody production and in association with liposomes their humoral response is not altered (Shek and Sabiston, 1982a; Latif, 1984). However, these reports are at variance with the study of van Rooijen and van Nieuwmegen (1983) who found IgM antibodies produced during primary immunization of BSA associated with liposomes. BSA molecules are associated with the liposome surface and it is possible that the surface-association is responsible for the observed IgM response. Haptens such as tripeptides (van Houte *et al.*, 1981) and sugars such as N-acetyl-D-glucosamine (Das *et al.*, 1984) and galactose (Sarkar and Das, 1984) result in the formation of IgM type of antibodies. Though this difference in the kind of antibody produced cannot be resolved at present it may be possible that the type of antigen has a significant role to play.

### Liposomes as carriers of additional immunomodulators

The immunoadjuvant activity of liposomes to associated antigens may be enhanced further by the incorporation of immunomodulators. Studies carried out with endotoxin and lipid A show an enhanced adjuvant effect to the antigen in association with liposomes (van Rooijen and van Nieuwmegen, 1980c; Dancey *et al.*, 1977). However, further increase in the adjuvant activity is observed when the antigen and the immunomodulator are incorporated in the same liposomes.

Another compound possessing immune potentiating activity is N-acetylmuramyl-L-alanyl-D-isoglutamine [muramyl dipeptide (MDP)] the minimal structural unit that can replace the immunoadjuvant activity of mycobacteria in CFA. Encapsulation of MDP in liposomes enhances its effectiveness in producing an immune response against antigen compared to the unencapsulated MDP (Chedid *et al.*, 1979). In addition, encapsulation of MDP in liposomes reduces the amount of drug needed for protection

against infections (Fraser-Smith *et al.*, 1983). Liposome-encapsulated MDP has been found to activate macrophages more efficiently *in vitro* than free MDP (Sone and Fidler, 1981). This property to activate macrophages has been further extended *in vivo* for the destruction of spontaneous lymph node and visceral metastasis (Fidler *et al.*, 1981). A more specific mediator involved in the activation of normal noncytotoxic cells of the macrophage-histocyte series to become cytotoxic against tumour cells is the soluble lymphokine referred to as macrophage-activating factor (MAF) (Churchill *et al.*, 1975). However, the *in vivo* administration of MAF to bring about systemic activation of macrophages has not been accomplished. Lymphokines have a short life and after injection into circulation they rapidly bind to serum proteins. Moreover, preparations of MAF are antigenic and therefore their repeated injections may not be possible. Encapsulation of MAF in liposomes prevents these undesirable side effects at the same time induces an efficient macrophage activation (Kleinerman *et al.*, 1983).

### Concluding remarks

The recent development in liposome research demonstrate the promising role of liposomes in immunology, in particular, as immunopotentiators and carriers of antigens. The major factors affecting the immunoadjuvant action of liposomes appear to be the charge of the vesicles and the site of association of the antigen with the lipid bilayer. The presence of sugar ligands on the surface of liposomes, further seem to regulate the production of antibodies to the associated antigens. However, for a clear understanding of the mechanism of action of liposomes further investigations are required. Still, the potential value of liposomes as immunological adjuvants has emerged as one of the brightest prospects among the many projected clinical applications of liposomes.

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## **Isolation of regulatory proteins affecting gene transcription**

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**Abstract.** This article considers ways in which various genes are regulated and the approaches that have been used to isolate the gene regulatory proteins involved. Isolation of regulatory proteins uses standard techniques for protein purification. The main problem in the isolation is not these techniques but rather assay procedures that permit detection of the regulatory protein during the course of isolation. For this purpose assays that are most useful are those that combine selectivity with sensitivity. In prokaryotes there is no better assay than that provided by the coupled transcription translation system. Plasmids containing regulatory genes can frequently be used as abundant sources of regulatory proteins and sometimes even permit the direct detection of the regulatory protein. In eukaryotes the situation is far more difficult because the isolation of regulatory genes is much more difficult. It seems likely that selective transcription and selective DNA binding will provide the most useful assays.

**Keywords.** Repressor; activator; transcription.

### **Isolation of regulatory proteins affecting gene transcription**

Gene expression begins with transcription, a process that is regulated by the natural affinity between the gene and the transcribing enzyme as well as a plethora of regulatory proteins that exert their effects directly or produce products that do. An important aspect of determining the mechanisms involved in gene regulation is the isolation of the regulatory proteins that mediate the level of transcription for different genes. In prokaryotes such isolations have become almost routine. In eukaryotes very few regulatory proteins have been isolated. Furthermore the isolations have usually been attended by peculiarly favorable circumstances that do not lend themselves to generally useful methodologies. The availability of methods for protein purification from crude extracts is not the problem. These abound, rather the critical problem is the availability of assay methods for detection of the regulatory protein in the crude extract which can be used to monitor enrichment during purification.

In this article I will review the ways in which various genes are regulated and the approaches that have been used to isolate the regulatory proteins involved. I will conclude by indicating the approaches which I feel are most likely to become general methods for isolating regulatory proteins from eukaryotes.

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Abbreviations used: PBS, Polymerase binding site; IPTG, isopropylthiogalactopyranoside; MTV, mammary tumor virus; DEX, dexamethasone; tk, thymidine kinase.



### Regulating transcription in *Escherichia coli*

Amongst the prokaryotes *E. coli* is by far the best understood. The *E. coli* chromosome contains about  $2 \times 10^9$  daltons ( $3 \times 10^6$  bps) of DNA, sufficient to code for approximately 2000 genes. Only about 5 % of the genome is highly active in transcription at any given time. The remainder of the genome is either silent or transcribing at a very low rate. When growth conditions change, some active genes are turned off and other inactive genes are turned on. The cell always retains its totipotency, so that within a short time (seconds to minutes in most cases), and given appropriate circumstances, any gene can be fully turned on. The maximal activity for transcription varies from gene to gene. For example, a fully expressing rRNA gene makes one copy per sec, a fully turned on  $\beta$ -galactosidase gene makes about one copy per min, and a fully turned on bicuculline synthase gene makes about one copy per 10 min. In the maximally repressed state, these genes express less than one transcript per 10 min. The level of transcription of any particular gene usually results from a complex series of control elements organized into a hierarchy that coordinates all the metabolic activities of the cell. For example, when the rRNA genes are highly active, so are the genes for ribosomal proteins, and these latter are regulated in such a way that stoichiometric amounts of most of the ribosomal proteins are produced. When glucose is abundant, most genes involved in processing more complex carbon sources are turned off in a process called catabolite repression; when the glucose supply is depleted and lactose is present then the genes involved in lactose catabolism are expressed.

### Basic mechanics of the transcription initiation process

The most commonly known way of regulating gene expression in bacteria involves controlling the rate of initiation of transcription (Rodriguez and Chamberlain, 1980). Prior to initiation of transcription, the RNA polymerase holoenzyme becomes attached to a 35- to 45-nucleotide segment of the DNA called the promoter. The affinity between DNA and polymerase is controlled by a sequence of bases in the DNA. Two main areas of contact have been recognized, one centered in the  $-35$  region of the promoter with the favored sequence of TTGACA and one centered in the  $-10$  region with the favored sequence TATAAT. These two hexanucleotide regions are referred to as polymerase binding site 1 (PBS1) and the polymerase binding site 2 (PBS2) respectively. From the time it first makes contact with the promoter to the time that it has achieved the proper orientation for initiation, the promoter-polymerase complex may go through several metastable states, the final conformation of this complex adopted immediately before initiation is referred to as the rapid-start complex or open promoter complex. In this state, the polymerase is in contact with PBS1 and PBS2, at about 11 base pairs from the  $-9$  to the  $+2$  positions at the origin of transcription; the bases are unpaired. Once the rapid-start complex has been formed, initiation of RNA synthesis is rapid, usually taking only a fraction of a second in the presence of ribonucleoside triphosphates. Since the spontaneous dissociation rate of the rapid-start complex is usually much longer than this, most rapid-start complexes once formed will initiate transcription after they have been formed. This means that for most promoters, the rate of formation of the rapid-start complex rather than the equilibrium constant of the complex is the

critical parameter that determines the activity of a promoter. The point of initiation of transcription (+1) is located eight or nine bases downstream from the center of the PBS2 site.

### Regulating initiation of transcription

The rate of initiation of transcription can be regulated in several ways, most of which are believed to influence the rate of formation of the rapid-start complex. The primary sequence of nucleotides in the promoter region is the first factor that should be considered. The closer this sequence is to the favored sequence (or consensus sequence) described above, the greater is the affinity of the polymerase for the promoter. For some promoters, negative supercoiling of the DNA serves as an appreciable stimulus to transcription. This probably results from the fact that negative supercoiling facilitates unwinding of the double helix, such as is required in the -9 to +2 region of the promoter in the rapid-start complex. The rate of initiation of transcription also can be altered by changes in the RNA polymerase structure. This structure can be altered by subunit replacement, subunit covalent modification, or small-molecule-induced allosteric transition. During sporulation of *Bacillus subtilis*, the  $\sigma$ -subunit of RNA polymerase is replaced, producing a change in the types of promoters recognized by the polymerase. In bacteriophage T4 infection of *E. coli*, the subunits of the polymerase become ribose-adenylated, lowering the affinity of the polymerase for bacterial promoters and raising the affinity for phage promoters. Although not finally proven, it is believed that binding of the guanosine tetraphosphate (ppGpp) to RNA polymerase changes its structure so that it has a greatly lowered affinity for rRNA and ribosomal protein promoters and at the same time a somewhat greater affinity for some other promoters. Finally, the rate of initiation of RNA synthesis can be controlled by auxiliary regulatory proteins that affect the rate of formation of the rapid-start complex in either a positive or a negative way; such regulatory proteins are known as activators or repressors, respectively. Some examples of these are discussed below.

### The lac operon

The story of the lac operon reveals one of the clearest and best-understood pictures of a gene regulatory mechanism (Zubay, 1980). To a considerable extent, studies on the lac operon have served (and will continue to serve) as a model for those studying other genetic regulatory mechanisms in protists and higher forms. Consequently we will consider it in some detail here. Significant events in elucidation of the concept and role of the operon have spanned a period of about 40 years. During this period advances in genetic and biochemical skills and advances in our understanding of the lac operon have gone hand in hand. Knowledge of the genetic processes of conjugation and transduction, coupled with elucidation of the basic mechanisms of DNA, RNA, and protein synthesis, have been essential to progress. More recently, cell free synthesis techniques, use of restriction enzymes for isolation and cloning of small discrete segments of DNA, and methods for determining nucleotide sequences have played important roles in advancing our knowledge of the lac operon.

The lac DNA is a region of the *E. coli* chromosome with a molecular weight of about

$4 \times 10^6$ , constituting about 0.2% of the *E. coli* genome. The DNA is separated into functional portions; the controlling elements of the operon, and the structural genes which code for the three proteins specified by the lac operon that are associated with lactose catabolism,  $\beta$ -galactosidase, lactose permease, and thiogalactosyl transacetylase.

### Enzyme induction

*E. coli* cells grown in the absence of a  $\beta$ -galactoside contain an average of 0.5 molecules of  $\beta$ -galactosidase per cell, whereas bacteria grown in the presence of an excess of a suitable inducer of the lac operon contain 1000 to 10,000 molecules per cell. Radioactive amino acid labelling was used to show that the increase in enzyme activity observed on induction results from *de novo* protein synthesis. On addition of either  $\beta$ -galactoside or inducer, enzyme activity increases at a rate proportional to the increase in total protein within the culture. Enzyme formation reaches its maximum rate within 3 min of addition of inducer at 37°C. After removal of inducer, enzyme synthesis ceases in about the same amount of time. A large number of compounds have been tested for their capacity to induce  $\beta$ -galactosidase. All inducers contain an intact unsubstituted galactosidic residue. Many compounds that are not substrates for  $\beta$ -galactosidase, such as thiogalactosides, make good inducers. Lactose, the natural substrate of the operon, is not the natural inducer *in vivo*. Rather allolactose, formed as an intermediate in lactose metabolism with the help of the very limited amount of  $\beta$ -galactosidase, present in uninduced cells, is believed to be the natural inducer. The three proteins encoded by the lac operon are coordinately induced, i.e. they are induced to the same extent by the same inducer. These results suggest that the receptor molecule for inducer is distinct from the structural components of the operon and that there is one site where inducer acts.

### Discovery of the repressor gene

Two distinct types of mutants have been observed in the lactose system. One class of mutants includes structural gene mutations: (i)  $\beta$ -galactosidase mutations ( $z^+ \rightarrow z^-$ ), expressed as the loss of the capacity to synthesize active  $\beta$ -galactosidase; (ii) permease mutations ( $y^+ \rightarrow y^-$ ), expressed as the loss of the capacity to concentrate lactose; and (iii) transacetylase mutations ( $a^+ \rightarrow a^-$ ), expressed as the loss of the capacity to form thiogalactoside transacetylase. The other class of mutations involves controlling elements of the operon, such as *i* gene constitutive mutants ( $i^+ \rightarrow i^-$ ), expressed as the loss of capacity to synthesize large amounts of  $\beta$ -galactosidase in the absence of inducer. Structural gene mutants usually affect only the enzyme in whose gene the alteration occurs. In contrast, most mutations in the *i* gene affect the amounts of  $\beta$ -galactosidase, permease, and transacetylase synthesized, but not their structures.

Some of the most informative early studies were performed with partial diploids (merodiploids) that contain the relevant genes on both cellular chromosome and *F* factor plasmid. Merodiploids of the type  $z^+y^-a^-/Fz^-y^+a^+$  or  $z^-y^+a^+/Fz^+y^-a^-$  are wild-type; that is, they behave normally; they metabolize lactose and form normal amounts of both  $\beta$ -galactosidase and transacetylase. This complete complementation between structural gene mutants indicates that they belong to independent genes on

cistrons. Most  $i^-$  mutants synthesize more  $\beta$ -galactosidase, permease and transacetylase than induced wild-type cells, but the ratio of  $\beta$ -galactosidase to acetylase is the same in these mutant cells as in induced wild type cells. Strongly suggesting the mechanism controlled by the  $i$  gene is related to inducer interaction.

The study of merodiploids of the types  $i^+z^-/Fi^-z^+$  or  $i^+a^-/Fi^-z^+$  demonstrated that the  $i^+$  inducible allele is dominant to the  $i^-$  constitutive allele and that it is active on the same chromosome (cis) or on a different chromosome (trans) with respect to both  $a^+$  and  $z^+$ . The fact that it acts in the trans position as well as the cis position shows that  $i$  gene mutations belong to an independent cistron, governing the expression of  $z$ ,  $y$ , and  $a$  through production of a diffusible cytoplasmic component. The dominance of the inducible to the constitutive allele suggests that the former corresponds to the active form of the  $i$  gene.

### Discovery of operator mutants

In the lac system, rare dominant constitutive mutants ( $o^c$ 's) were isolated by selecting for constitutive expression of  $\beta$ -galactosidase in cells diploid for the lac region, including the  $i$  gene, thus virtually eliminating the much more frequently occurring recessive ( $i^-$ ) constitutive mutants (since two copies of  $i$  gene are present in such cells, both  $i$  genes would have to mutate simultaneously to give a constitutive phenotype). If the probability of an  $i^+ \rightarrow i^-$  mutation is  $10^{-6}$ , the probability of two such simultaneous events in the same cell would be  $10^{-12}$ . By recombination, the  $o$  mutants were mapped in the lac region between the  $i$  and  $z$  loci. Genetic and biochemical evidence exists showing that the  $o$  locus is adjacent to but distinct from the  $z$  gene. The  $o$  mutations affect the quantity of  $\beta$ -galactosidase synthesized but not its structure.

In merodiploids of the type  $o^c/o^+$ , galactosidase and transacetylase are constitutively synthesized, showing that the  $o^c$  mutation is dominant. In merodiploids of the type  $o^c z^+/o^+ z^-$  and  $o^c z^-/o^+ z^+$ ,  $o^c$  is dominant in the former but recessive in the latter for galactosidase expression. Thus the  $o^c$  mutation is only dominant in the cis position. From this evidence, Jacob and Monod (1961) inferred that the  $o^+ \rightarrow o^c$  mutations correspond to a modification of the specific repressor-accepting structure of the operator. The location of these mutants identifies the operator locus.

### The operon hypothesis

The behavior of the various mutations discussed above led Jacob and Monod (1961) to propose a model for the regulation of protein synthesis. The genetic elements of this model consist of a structural gene or genes, a regulator gene, and an operator locus. The structural gene produces a messenger RNA molecule that serves as the template for protein synthesis. The regulator gene produces a repressor that can interact with the operator locus. The operator is always adjacent to the structural genes it controls. The operator and its associated structural genes are referred to as the operon. Combination of the repressor molecule with the operator prevents the structural gene(s) from synthesizing messenger RNA. In induction, the inducer (antirepressor) is thought to combine with the repressor to prevent its interaction with the operator. This operon

hypothesis provided a tremendous stimulus for investigations directed toward understanding not only the lac system, but other genetic regulatory systems as well.

### Isolation and properties of the *i* gene repressor

According to the operon hypothesis, antirepressor is supposed to combine with repressor. Taking advantage of this fact, Gilbert and Muller-Hill (1966) used [ $^{14}\text{C}$ ] labelled isopropylthiogalactopyranoside (IPTG), one of the strongest known antirepressors, to monitor repressor purification from a crude cell extract. Unlike the natural inducer allolactose, IPTG not only binds strongly to repressor, but it is completely stable in *E. coli* cells or crude extracts. A crude cell-free extract was fractionated by standard protein purification procedures, and the fraction containing the repressor that binds IPTG was found. Binding of any particular extract was measured by equilibrium dialysis. When using this procedure, the extract was placed inside a semipermeable dialysis bag that was impermeable to protein but permeable to IPTG. This was allowed to equilibrate with the external solution by gentle agitation in the presence of a buffer containing radioactive inducer. At the end of the dialysis, a certain concentration of free inducer ( $I_f$ ) should be detectable outside the bag. The inducer concentration inside the bag should exceed that outside by the amount that is bound to the protein ( $I_b$ ). The strength of binding of repressor to IPTG could also be determined by equilibrium dialysis. When the ratio  $I_b : I_f$  is plotted versus  $I_b$ , the slope should equal the negative of the formation constant for binding  $K_f$ , where  $K_f$  is defined by the equation,

$$K_f = \frac{(\text{repressor} - \text{IPTG})}{(\text{repressor})(\text{IPTG})}; \quad K_d (\text{dissociation constant}) = \frac{1}{K_f}.$$

The formation constant for this complex using wild-type repressor is approximately  $10^6 \text{ M}^{-1}$ ; the  $K_f$  for allolactose binding to repressor is about 100 times higher. Variants of wild-type repressor purified to the same degree also were examined. The mutant repressor  $i^+$ , which was isolated from strains that are easier to induce, had about double this affinity. The repressor encoded by  $i^s$ , isolated from noninducible strains showed no affinity for IPTG. Typical  $i^-$ -mutant repressors bound IPTG normally but were presumed to have a defective site for binding to DNA.

The binding of purified repressor to DNA was demonstrated by the membrane-filter binding technique. This technique is based on the fact that most proteins bind to nitrocellulose filter membranes, but DNA does not. However, DNA complexed to protein does bind to the membrane filter. With this technique radioactively labelled DNA is used in conjunction with unlabelled purified repressor. A solution containing labelled  $\lambda$ lac DNA (i.e.  $\lambda$ DNA containing the lac operon) with or without added repressor was passed through the membrane filter with the help of mild suction. Only when repressor was present was the label retained by the filter. If DNA or if  $\lambda$ lac DNA containing an  $\sigma^c$  mutant was used the DNA was not retained by the filter.

The RNA polymerase and repressor binding sites have considerable overlap, so that the binding of one protein precludes the binding of the other. Whereas there are other DNA sequences where the repressor might bind to prevent initiation, this may be the most common situation.

### Isolation and properties of the catabolite gene activator protein cap

When *E. coli* cells are grown in the presence of glucose and lactose as the sole carbon sources, the cells will first utilize glucose and afterwards utilize the lactose. This phenomenon is referred to as diauxic growth; it reflects a type of regulation not anticipated at the time the operon model was proposed. Careful analysis of the cells has shown that during the glucose phase of growth, the lac operon is poorly expressed and induction of the operon must occur before the lactose can be utilized. Repression of lac operon expression by glucose is known by the general term catabolite repression. Enzymes of the glycolytic pathway that directly utilize glucose are always present in the cell. The extra enzymes of the lac operon are not needed as long as this more directly utilizable carbon source is available. Many genes in *E. coli* associated with catabolism are subject to this type of repression for the same reason. A turning point in our understanding of catabolite repression was provided by the finding that the intracellular cAMP level in *E. coli* is drastically lowered in the presence of glucose. Subsequently it was shown that large quantities of cAMP in the growth medium could partially reverse the glucose catabolite repression effect on the lac operon. The stimulatory effect of cAMP on genes subject to catabolite repression was demonstrated more directly in a cell-free system in our laboratory. The system used contained  $\lambda$  lac DNA, a cell-free extract of *E. coli*, and the substrates and other low-molecular weight components necessary for transcription of the DNA and translation of the resulting messenger RNA (mRNA). In this so-called coupled system for transcription and translation it was shown that cAMP stimulated  $\beta$ -galactosidase synthesis about 30-fold. Subsequently, mutants of *E. coli* that were permanently catabolite-repressed were isolated by Schwarty and Beckwith (1970). The phenotype of such mutants was that they were incapable of expressing a number of genes that required cAMP for activation. These mutants fell into two categories, those which could be phenotypically corrected by growing in the presence of cAMP and those which could not. The first class of mutants was defective in the synthesis of cAMP, and the latter class of mutants was defective in the protein(s) with which cAMP interacts to bring about stimulation of  $\beta$ -galactosidase synthesis as well as the synthesis of other proteins whose synthesis is subject to catabolite repression. Further work showed that these two types of mutants originate in the genes *cya* and *crp*, respectively. When the cell-free system was made from extracts of *crp*-cells, it produced a low level of  $\beta$ -galactosidase that was not stimulated by adding cAMP. The defect could be corrected by adding soluble protein from *crp*<sup>+</sup> cells. Making use of this *in vitro* complementation assay, it was possible to fractionate the *crp*<sup>+</sup> cells and isolate a single protein termed CAP that was responsible for the activity. At moderate salt concentrations, CAP interacts with cAMP with a formation constant,  $K_f$ , of  $0.6 \times 10^5 \text{ M}^{-1}$ , where  $K_f$  is defined by the equation

$$K_f = \frac{(\text{CAP} - \text{cAMP})}{(\text{CAP})(\text{cAMP})}$$

The structural alterations produced in CAP by cAMP binding apparently alter its conformation so that it can form a strong complex with DNA. CAP binds preferentially to the lac promoter region, although the selectivity for preferential binding to the specific site is substantially less than is the case for lac repressor. It was demonstrated

from a series of genetic deletions that the site necessary for CAP stimulation of the lac operon is in the -50 to -80 base-pair region of the promoter.

The overall strategy in creating a promoter sequence responsive to cAMP-CAP activation could be summarized as follows. The nucleotide sequence in the RNA polymerase binding site is adjusted so that polymerase by itself produces a low level of transcription. An adjacent site for binding CAP is created so that when CAP is binding the additional affinity contributed by favorable contacts between the CAP and the polymerase convert this into a high-level promoter.

### **Behavior of some other genes important in catabolism**

The cAMP-CAP activator is an essential component for regulating a wide variety of genes involved in catabolism. It usually functions in series with a gene-specific regulator so that any particular gene subject to cAMP-CAP activation will only be fully expressed if both control switches are in the "on" position. For example, the galactose operon encoding enzymes for galactose catabolism is regulated by cAMP-CAP as well as by a specific repressor that is antagonized by galactose. The arabinose operon also uses the pleiotropic cAMP-CAP activator and a specific regulator.

### **Regulation of genes involved in anabolic processes. The tryptophan operon**

Amino acid synthesis in *E. coli* is regulated at the level of transcription and through the modulation of certain enzyme activities located at branch points in the various biosynthetic pathways. Of all the genes concerned with anabolic processes, the genes involved in tryptophan biosynthesis are the best understood. This is mostly a result of the efforts of Zurawski *et al.* (1978) who have used a wide variety of genetic and biochemical techniques to probe the complexities of this system (Zubay, 1980). Wild-type *E. coli* has the capacity to synthesize tryptophan from simpler substrates, but does so only when the amino acid is not available in adequate amounts from the external growth medium. A lowering of tryptophan stimulates synthesis of the messenger RNA and the five polypeptide chains associated with the biosynthesis of tryptophan from chorismic acid. The five contiguous structural genes are transcribed as a single polycistronic messenger RNA. Initiation of transcription is regulated by the interaction of the tryptophan aporepressor, the protein product of the *trpR* gene, with its target site on the DNA, the *trp* operator. Binding of L-tryptophan to the aporepressor causes a structural alteration essential for strong specific binding to the *trpO* locus. Like *lac* repressor, the *trp* repressor binds at a site that overlaps the RNA polymerase binding site. The most significant difference between the action of the *trp* and *lac* repressors relates to the function of the small-molecule effector. In the case of *lac*, the effector molecule allolactose acts as an antirepressor, causing the release of repressor from the operator; in the case of *trp*, the effector molecule L-tryptophan acts as a corepressor, stimulating the binding of repressor to the operator. It should be obvious that the difference in action of these small-molecule effectors, whose concentrations dictate the level of operon activity, is well-suited to the different metabolic needs of the cell satisfied by the two operons.

### Control of stable rRNA synthesis by the *rel* gene

Some small molecule effectors may interact directly with the RNA polymerase. This appears to be the case in the control of rRNA synthesis by ppGpp. *E. coli* cells under conditions of rapid growth contain about  $10^4$  ribosomes. The maximum rate of reinitiation at the ribosomal gene promoter is about 1 per sec. In rapid growth *E. coli* can duplicate once every 20 min, which would allow for the synthesis of only about 1200 molecules of rRNA if there were only one gene for ribosomal RNA. In fact, there are seven copies for ribosomal RNA in the bacterial chromosome, which makes it possible for rRNA synthesis to maintain the necessary pace under conditions of rapid growth. The rRNA operons are dispersed at seven locations around the circular *E. coli* chromosome. Each operon is transcribed into one long transcript that is processed into a single 16S, 23S, 5S, and one or more than one transfer RNA. The order of the genes in the operon starting from the initiation site for transcription is 16S, 4S, 23S, and 5S. In some of the operons, additional 4S genes for tRNA are located downstream from the 5S gene. All the known ribosomal RNA operons appear to have two promoters located in tandem.

As stated earlier, rRNA synthesis is usually maintained at a rate that is proportional to the gross rate of protein synthesis. In a normal wild-type cell, when protein synthesis is limited, *e.g.* by amino acid availability, there is a rapid rise in ppGpp concentration from about 50 to 500  $\mu$ M. Concomitantly, there is an abrupt cessation of rRNA synthesis. This is part of the syndrome known as the stringent response. If amino acids are reintroduced into the growth medium, the ppGpp concentration falls rapidly (half-life about 20s), and the rate of rRNA synthesis rises. In a *relA* mutant cell, neither the rapid rise in ppGpp concentration nor the cessation of rRNA synthesis are seen when amino acids are removed. The *relA* gene encodes a protein that is involved in ppGpp synthesis. Strong support that ppGpp directly inhibits rRNA synthesis comes from cell-free synthesis studies in which the DNA-directed synthesis of rRNA has been shown to be strongly and selectively inhibited by high levels of ppGpp.

The synthesis of ppGpp has been studied both in crude cell-free extracts of *E. coli* and in a partially purified system to determine what factors influence its rate of synthesis. It was found that ppGpp is synthesized on the ribosome from GTP in the presence of the protein encoded by the wild-type *relA* gene. Maximum synthesis occurs in the presence of ribosomes associated with mRNA and uncharged tRNA with anticodons specified by the mRNA. If the uncharged tRNA bound to the ribosome acceptor site (A site) is replaced by charged tRNA, the rate of ppGpp is greatly lowered. If uncharged tRNA anticodons are not complementary to mRNA codons exposed on the ribosome for protein synthesis, ppGpp synthesis does not occur. This set of observations suggests the mechanism whereby amino acid charging of tRNA controls the rate of rRNA synthesis. First, uncharged tRNA that is codon-specific for the exposed codons on the mRNA becomes bound to the ribosome acceptor site, creating a situation unfavorable for protein synthesis but favorable for ppGpp formation. Second, the ppGpp diffuses and binds to RNA polymerase or other factors involved in rRNA transcription. Although it has been hypothesized that ppGpp acts by binding to RNA polymerase final proof that this mechanism is correct will probably require the isolation of RNA polymerase mutants that are not affected by ppGpp.



### Regulation of gene expression in bacterial viruses: The bacteriophage $\lambda$

The strategies used by bacterial viruses to regulate gene expression are quite different. Nevertheless the genetic and biochemical approaches used to investigate the situation have been quite similar (Hershey, 1971; Hendrix *et al.*, 1983). Temperate viruses like  $\lambda$  can adopt either an active lytic state or a dormant prophage state. In the prophage state viral chromosomes are found at a low copy number in a host-integrated form. In the lytic state temperate phage chromosomes duplicate rapidly so that within about an hour they kill the host cell, which usually lyses releasing infectious particles. In the prophage state  $\lambda$  appears to utilize the unmodified host RNA polymerase. Viruses that enter the irreversible lytic cycle, whether they are temperate or virulent, either modify or replace the host polymerase to enhance their own transcription.

Immediately after the linear duplex  $\lambda$  DNA chromosome enters an *E. coli* cell it becomes circularized and supercoiled by host enzymes. Early transcription by  $\lambda$  is catalyzed by the unmodified host RNA polymerase. The promoters active at this time include  $P_R$ ,  $P_L$  and  $P_{R'}$ . Terminators  $t_{R1}$ ,  $t_{R2}$ ,  $t_{L1}$  and  $t_{65}$  prevent more than limited transcription from these promoters. The rates of synthesis of various early gene products determines which route  $\lambda$  follows.

#### Events favoring lysogeny

Early transcription from  $P_R$  leads to the transcription of the *cII* gene which encodes a regulatory protein. *cII* is an activator protein like CAP. Unlike CAP it requires no small molecule co-activator. High concentrations of *cII* favor the establishment of lysogeny. This is because *cII* stimulates transcription from  $P_{RE}$  and  $P_{INT}$ . Transcription from  $P_{RE}$  leads to the synthesis of *cI* which is a repressor that inhibits transcription from both  $P_R$  and  $P_L$ . Low to moderate concentrations of the *cI* protein also stimulates the  $P_{RM}$  promoter leading to further synthesis of the *cI* protein. *cII*-stimulated transcription from  $P_{INT}$  leads to synthesis of the *INT* protein required for integration. If sufficient *cII* protein is made, lysogeny will result because the subsequent synthesis of *cI* will shut down early right and early left transcription from  $P_R$  and  $P_L$ , a requirement for the lytic cycle. The *cII*-stimulated synthesis of *INT* protein provides the essential phage-encoded enzyme for integration of the  $\lambda$  DNA into the host chromosome.

#### Maintenance of the lysogenic state

The lysogenic state is maintained by moderate concentrations of the *cI* protein (about 100 molecules per cell). Under these conditions transcription from  $P_R$  and  $P_L$  is inhibited. Whereas low or moderate concentrations of the *cI* protein stimulate synthesis of the *cI* protein, high concentrations of *cI* protein inhibits such synthesis. Thus *cI* protein acts as a modulator of its own synthesis, stimulating the synthesis of sufficient levels of itself to stabilize the lysogenic state but preventing excessive levels of synthesis that might make it difficult to activate the prophage under the desired conditions. This complex action of the *cI* protein is explained by the presence of three adjacently located binding sites that bind *cI* protein with different affinities. In the lysogenic state synthesis

of cI protein from the  $P_{RE}$  promoter does not occur because of the lack of the cII protein.

### Activation of the prophage and other events leading to the lytic cycle

Activation of  $\lambda$  prophage requires proteolytic cleavage of the cI repressor. This reaction is catalyzed by a highly specific protease function of the recA protein which is activated under conditions leading to DNA damage. Once the cI protein has been destroyed the  $P_R$  and  $P_L$  promoters become active leading to a cascade of events that encourages the lytic cycle. Early during activation, the prophage becomes excised and circularized. Subsequent events in the lytic cycle are similar for the activated prophage or  $\lambda$  DNA infecting the cell from without.

The overall strategy of events occurring during the lytic cycle is similar for most lytic phage. Phage encoded products are made as they are needed and cease to be made when they are present in sufficient concentrations or no longer needed. Enzymes required for phage recombination and replication are made early in the lytic cycle. Lysis proteins and structural proteins are made late.

In  $\lambda$  most of the synthesis is regulated by the availability of specific transcripts. The concentrations of the various messengers is regulated by six  $\lambda$ -encoded regulatory proteins: cI, cII, cIII, cro, N and Q.

As stated previously transcription becomes active from  $P_L$  (leftwards) and  $P_R$  (rightwards) in the absence of cI. The first gene to be transcribed from rightward synthesis is *cro* which encodes a repressor that inhibits cI synthesis from  $P_{RM}$ . During the lytic cycle the number of DNA copies increases so that even a low activity of the  $P_{RM}$  promoter could lead to enough cI protein synthesis to interfere with the early right and early left transcription. The *cro* protein prevents this from happening. Later during the lytic cycle  $P_R$  activity declines as a result of the elevated concentration of *cro*, which leads to the binding of more than one *cro* protein (3) between the  $P_{RM}$  and  $P_R$  promoters. Early leftward transcription leads to the synthesis of the N protein. In the absence of N, early transcription stops at the  $t_{L1}$ ,  $t_{R1}$  and  $t_{R2}$  promoters. In the presence of N protein attenuation occurs at these promoters as a result of N binding to polymerase. This permits the elongation of the leftward and rightward transcripts resulting in the synthesis of additional proteins. Even in the presence of N protein, rightward transcription stops at the  $t_{65}$  terminator preventing synthesis of late gene products required for cell lysis and head and tail structural proteins. Late rightward transcription does not occur until the Q protein has been made. This regulatory protein, like the N protein, is an antiterminator that functions by binding to the polymerase. Q protein eliminates termination at the  $t_{65}$  promoter so that transcription initiating at either  $P_R$  or  $P_L$  continues through the genes required for late functions.

At this point it is appropriate to make a general comparison between the types of regulatory proteins used by the host *E. coli* cell and by the  $\lambda$  phage. In both systems one finds repressors and activators which function by binding to the DNA near the polymerase binding site. The nature of the repressors and activators of the phage differ in major respects. Their action is not modulated by small molecule effectors. Rather their action is a function of their presence or absence. For example the *E. coli* lac repressor is reversibly inactivated by allolactose. To inactivate the  $\lambda$ cI repressor

proteolysis is required. The *E. coli* CAP protein is activated by cAMP. No activator is required for  $\lambda$ cII activation. Indeed cII protein is inherently unstable with a short half life. The main function of cIII appears to be to stabilize the cII protein. Host encoded antiterminators that function like N and Q probably also exist for the rRNA genes in bacteria. In bacteria regulatory proteins for most genes appear to be present most of the time. Whether or not they are active depends upon the concentration of small molecule effectors like cAMP or allolactose. This probably results from the desirability to maintain most bacterial genes in an "always prepared" state so that they can be readily expressed when needed or rapidly turned off when they are not needed. By contrast phage genes are called upon to express only at fixed times during the lytic cycle by an irreversible process which permits the concentrations of the regulatory proteins to vary over wide ranges. There is no need for small molecule effectors to modulate the activity; rather the activity of the genes is controlled by the concentrations of the regulatory protein itself.

### Regulation of gene expression in eukaryotes

There are some sharp distinctions between the ways in which a prokaryote like *E. coli* and a unicellular eukaryote like the yeast *S. cerevisiae* transcribe and translate that have led to clearcut differences between the types of gene regulatory mechanisms used by these organisms. For example splicing of mRNA is unique to eukaryotes whereas the ability to translate polycistronic messages is unique to prokaryotes. There is a single transcribing enzyme in prokaryotes for all genes whereas in eukaryotes the task of transcribing is apportioned to three major enzymes. Pol I, Pol II and Pol III. In comparing simple eukaryotes like yeast with complex eukaryotes like insects and mammals such sharp functional distinctions have not been found. For example, consider the phenomenon of cellular differentiation. This is omnipresent in higher forms but yeast also shows differentiation into three cells types,  $a$  and  $\alpha$  haploid types and  $a/\alpha$  diploids. The same is true with hormones which provide a major mechanism for intracellular communication between the cells of higher forms. Haploid yeast cells do in fact secrete pheromones which impinge on cells of the opposite mating type and change their pattern of gene expression even prior to the cell-to-cell contact that leads to diploidization. Such comparisons have made simpler eukaryotes like yeast extremely valuable as vehicles for studying cellular differentiation and hormonal control but they do not eliminate the need to study these processes directly in higher forms if we are to understand them in any detail.

### Chromosome structure varies with gene activity

All eukaryotic DNA exists in the form of chromatin which contains an approximately equal weight of DNA and the five basic histone proteins. In normal interphase cells chromatin can exist in either a compact form known as heterochromatin or a swollen form known as euchromatin. Most nuclei contain both types of chromatin. The heterochromatin is generally concentrated around the nucleolus and the inside of the nuclear envelope. Some chromosomes or parts of chromosomes are heterochromatic

all the time (constitutive heterochromatin) others are heterochromatic only during certain times of the cell cycle or in certain cell types (facultative heterochromatin). The ratio of euchromatin to heterochromatin increases with increasing protein synthetic activity. Many studies indicate that euchromatin is relatively active in RNA synthesis and heterochromatin relatively inactive. Whereas the swollen euchromatic state seems to be necessary for a high degree of transcription for most genes it is not sufficient. Several biochemical changes accompany the gross morphological differences associated with the transition between heterochromatin and euchromatin. These changes include chemical modification of histones, a redistribution of nucleosomes along the DNA duplex, an alteration in the pattern of non-histone chromosomal protein binding and chemical modification of the DNA. At the present time most of these changes can only be discussed in a descriptive manner because we do not know their causes or the way they influence transcription.

### **Viruses viewed as model systems for studying the mechanisms of regulation of gene expression in eukaryotes: Observations on SV40 virus**

DNA viruses that use the host transcription and translation machinery have provided relatively simple systems for exploring the mechanisms used for regulating gene expression. One of the systems exploited most frequently for such purposes is the SV40 virus (Tooze, 1981). It has been possible to obtain mutants in all of the viral genes and to determine the effects that these mutants have on viral gene expression. The SV40 chromosome is a circular duplex molecule with about 5200 base pairs. Viral RNA synthesis is divided into early and late phases. The mRNAs encoding the two early proteins called small *t* and big *T* antigen are identical at their 5' and 3' ends but differ in their patterns of splicing. The large *T* antigen, molecular weight 88,000, binds as a tetramer specifically to a region of SV40 DNA that contains the origin of replication and the early transcriptional promoter. Similar to the *cI* and *cro* binding sites in  $\lambda$ , the locus for *T* antigen binding contains three binding sites. Binding site I has the highest affinity and binding site III the lowest affinity for the large *T* antigen. As might be suspected from the location of the binding sites, *T* antigen inhibits early transcription. It has been possible to demonstrate the inhibitory effect on transcription both *in vivo* and *in vitro*. The physiological significance of this inhibition has been demonstrated most clearly with temperature sensitive mutants for large *T*. Such mutants greatly over-produce early messenger at elevated non permissive temperatures. The effect of *T* antigen appears to be similar to the action of a transcription repressor in a prokaryotic system.

In prokaryotes the promoter for initiation of transcription is always located as a cluster of sequences immediately upstream of the gene encoding sequences. In eukaryotes the situation is frequently more complex and in most cases poorly understood (Manley, 1983). Most eukaryotic genes that are transcribed by Pol II contain the sequence TATA about 27 bases upstream from the transcription start site (position -27). Further upstream (-70 to -80) some promoters contain the sequence CAAT. The importance of the TATA sequence for some genes has been shown with mutants. Base replacements or deletions of the TATA box frequently result in drastic

reductions in transcription. For some genes deletion of the TATA box does not abolish transcription *in vivo*. Indeed mutants of SV40 lacking a TATA box transcribe effectively from the early region using a new and more heterogeneous set of start sites. The function of the TATA box for such genes appears to be to fix the precise site for transcription starts rather than to control the amount of transcription.

Between 70–110 bps upstream of the transcription initiation site in SV40 there is a 21 bp tandem repeat sequence required for early transcription *in vivo*. *In vitro* studies in Tjian's laboratory have led to the isolation of a host protein called Sp1 which produces a 40-fold stimulation of pol II catalyzed early transcription (Dyran and Tjian, 1983). Footprinting studies have shown that this factor binds specifically to the 21 bp tandem repeat sequence in SV40.

Further upstream from the normal transcription start site for early SV40 transcription there is a tandemly repeated 72 base pair sequence (–116 to –188 and –189 to –261 from the start site). Removal of one of these sequences has no effect on transcription *in vivo*. But removal of both of these 72 bp sequences results in a drastic lowering of early transcription. The requirement for the 72 bp sequence cannot be mimicked in a cell-free *in vitro* system indicating a serious lack of one or more factors in currently used *in vitro* systems. One of the major differences between *in vivo* and *in vitro* systems as far as the DNA is concerned is the lack of a nucleosome structure in the latter. Either this or other aspects of the tertiary structure may be to blame for this lack of parallel behavior. Further, *in vivo* observations indicate most surprisingly that the precise position or orientation of the 72 bp segment is not critical (Wasylyk *et al.*, 1983). This so-called enhancer sequence may be moved further upstream or it may be inverted without affecting its potency. This insensitivity of the enhancer to position or orientation makes it unique amongst promoter elements. Enhancers with different sequences have been found for many other eukaryotic genes.

### The 5S genes in frogs

In eukaryotes one often finds groups of genes that are functionally related. Frequently these so-called gene families are also structurally and evolutionarily related. Investigation of different gene families have been greatly aided by recombinant DNA technology which has permitted the isolation from the nuclear DNA of specific members of such families on small hybrid plasmids. This has permitted a detailed investigation of their sequence arrangements and in some cases their mode of transcription. Five of the best understood gene families are: (i) the 5S genes of frogs, (ii) the ribosomal genes, (iii) the histone genes, (iv) the globin genes, and (v) the immunoglobulin genes. Only in the case of the 5S genes is their significant information on relevant regulatory proteins (Sakonju and Brown, 1982).

The 5S ribosomal RNA genes of most eukaryotes are present in multiple gene copies and are organized in simple tandem multigene families. In amphibians the 5S RNA genes are organized into different multigene families which are under developmental control. For instance, in the African clawed toad, *Xenopus laevis*, the normal haploid genome contains about 20,000 copies of the 5S RNA genes organized into three different multigenic families. The two major families comprising about 98 % of the total

5S RNA genes are expressed only in growing oocytes. The third family containing about 400 copies are active in both somatic cells and growing oocytes. This developmental control is useful to the oocyte, permitting it to accumulate 5S RNA at rates 1000-fold higher than is possible in somatic cells.

Purified 5S DNA is transcribed accurately when injected into the nuclei of living oocytes. It may also be transcribed by extracts of these same nuclei using either high-molecular weight genomic 5S DNA or individual repeating units of 5S DNA joined to bacterial plasmids and cloned in *E. coli*. The 5S genes are transcribed by RNA polymerase III. One cloned 5S DNA of *Xenopus borealis* somatic 5S DNA was subject to a more detailed analysis of the sequences that influence its accurate transcription. For this purpose a collection of plasmids containing deletions of the 5S gene and its flanking regions was constructed. Two deletion series were made, one removing increasing amounts of DNA from the 5' end and another removing increasing amounts of DNA from the 3' end of the gene. Individual repeating units of 5S DNA were accurately transcribed into 5S RNA demonstrating that the signals for initiation and termination are present in each repeating unit rather than at one end of a tandem array of genes. Studies of deletions from the 5' end showed that the entire flanking sequence could be removed without interfering with proper initiation. As much as the first third of the gene could be removed without interfering appreciably with accurate initiation. In this event initiation started at a comparable location in the plasmid DNA. Deletions proceeding downstream beyond the +50 residue showed a loss of proper initiation. When deletions were made from the 3' end, initiation was not affected until the deletion went upstream beyond to +87 residue. The surprising but inescapable conclusion from these observations is that the control region for initiation is inside the gene and extends approximately from the 50th to the 87th residue. Internal promoters have thus far not been found in prokaryotes or in genes transcribed by the eukaryotic Pol II polymerase. Further studies have shown that it is not the RNA polymerase III that binds in the +50 to +87 region but rather a regulatory protein which influences the binding of Pol III at an upstream location. Thus a 40,000 dalton protein has been purified which is required for accurate *in vitro* transcription. This 40 kd protein was isolated from soluble extracts of *X. laevis* oocytes by monitoring the ability of the factor to stimulate transcription of exogenous 5S genes. When oocyte nuclear extracts were pretreated with antibody to the 40 kd protein they were no longer competent in initiating *in vitro* transcription from the 5S gene.

The approximate location of the binding site for the 40 kd protein to the 5S gene was determined by the so-called footprinting technique. For this purpose, DNA fragments containing the 5S gene were 5' end labelled. These were mixed with the 40 kd protein followed by partial DNaseI digestion. The resulting DNA fragments were electrophoresed on a polyacrylamide gel and afterwards the gel was autoradiographed. Regions of the DNA that were protected from DNase attack by 40 kd protein binding appear as a blank spot (footprint) on the autoradiogram. The protected region is situated between the 40th and the 90th residue in reasonable agreement with the hypothesis that the 40 kd protein binds specifically to this region of the 5S gene.

Competition binding experiments where both somatic and oocyte 5S DNAs are exposed to limited amounts of the 40 kd protein show that the regulatory protein binds considerably more firmly to the somatic 5S genes. This almost certainly explains why

the latter are uniquely active in somatic tissues when the amounts of 40 kd protein are much lower.

### Steroid hormone receptors

Steroid hormone receptors are believed to constitute a major class of gene activating proteins in higher eukaryotes. They are the only class of gene regulatory proteins in eukaryotes for which a systematic procedure for isolation has been developed. Despite this little is known about how they function. A particular steroid receptor is only found in a so-called target cell where it may be present in from  $10^3$  to  $10^5$  copies. Steroid hormones can have dramatic effects on the concentrations of mRNAs within a target cell. For example, in chicken oviduct cells ovalbumin mRNA increases from about 50,000 molecules per cell in response to estrogens. Oviduct cells respond positively to estrogens because they contain the estradiol receptor. Other cells lacking this receptor are unaffected by estrogens.

Steroid hormone receptors can be purified from target tissue using binding to an appropriate radioactive steroid hormone to monitor purification. The strong specific binding between receptor and hormone may also be used as a major step in the purification process. So-called affinity columns containing covalently attached steroid hormone selectively retain hormone receptors present in crude extracts.

Hormone receptors that are complexed with the hormone have an increased affinity for double-stranded DNA. However, the binding is of low affinity ( $K_d \cong 10^{-4}$  M) and appears to be sequence-independent. It is generally assumed that there is a small number of DNA sequences within the genome that bind receptors with high affinity ( $K_d \cong 10^{-10}$  M) but they are not observed in most DNA-binding assays because of the vast number of low affinity sites. Recently strong specific binding sites for the glucocorticoid receptor have been detected by using discrete segments of DNA. This work was initiated in Yamamoto's laboratory through studies on cultured rat hepatoma (HT) cells bearing chromosomally integrated murine mammary tumor virus (MTV) genomes introduced by infection (Payvar *et al.*, 1982). In such cells glucocorticoid hormones stimulate the rate of viral gene transcription in a receptor-dependent manner. Exposure to  $1 \mu\text{M}$  dexamethasone (DEX) increases the yield of virus specific RNAs. Steroid induced transcription varies from 0 to 50 fold as a function of the chromosomal position of provirus integration. Some integrations show a high basal level of the viral RNA synthesis, others do so only when treated with the steroid hormones. Using a series of restriction fragments of the proviral DNA several preferred binding sites for the glucocorticoid receptor have been found. Three of these are located in each of the so-called LTR segments which are tandemly oriented at the ends of the proviral DNA. The upstream binding sites for the glucocorticoid receptor map 110 to 449 bp from the main initiation site for transcription. Fusion of the LTR segment to the herpes simple virus thymidine kinase (tk) gene results in as much as a 50-fold stimulation of tk specific RNA transcription by DEX. Comparison of several different constructions indicates that the location and orientation of this glucocorticoid response element relative to the transcription start site is not rigidly constrained. This flexibility with respect to location suggests that the glucocorticoid response element may function like the enhancer.

element found in SV40 and a number of other genes, both cellular and viral. It remains to be seen if other steroid-responsive elements elicit their action through an enhancer-like element or whether some of them also behave as typical positive control elements in bacteria like CAP, the catabolite gene activator protein.

Thus far it has not been possible to demonstrate stimulation of transcription with steroid hormone receptors *in vitro*. Since the glucocorticoid receptor is believed to interact with an enhancer element this may be related to the general difficulty of demonstrating enhancer element effects on transcription *in vitro* (see the discussion on SV40 above).

### **An overview of different strategies used to regulate transcription**

In the foregoing discussion I have attempted to review some major patterns of control narrowing the discussion to control mechanisms that operate at the level of transcription. Aside from those forms of regulation that involve DNA rearrangements or DNA amplification, transcriptional regulation always involves processes that influence the affinity between RNA polymerase and the template either at the site of initiation or at a downstream location. The factors involved include the sequence of bases, particularly in the promoter region of the gene. As we have seen regulatory proteins can influence the interaction of polymerase with the gene in two general ways: (i) by modification of the RNA polymerase structure in a way that changes its affinity for the gene or (ii) by interaction with the DNA so as to change the affinity of the polymerase for the gene.

In prokaryotes DNA control sites important in transcription are always (at least so far as we know) contiguous with the gene being transcribed. Furthermore gene activity is always highly reversible so that merely by changing growth conditions genes may be rapidly turned on or turned off. In eukaryotes the situation is frequently more complex. The location of a gene within the chromosome is often a factor affecting its expression. Enhancers can greatly augment expression even when they are located at some distance from the gene in question. Activation or repression of particular genes frequently requires cell division to occur. Some genes appear to be permanently turned off in certain types of differentiated cells. These complex aspects of eukaryotic gene expression are difficult to approach at the present time especially since regulatory genes cannot be readily identified as they are in prokaryotes like *E. coli* or in simple eukaryotes like the yeast *S. cerevisiae*.

### **Methods for isolating gene regulatory proteins**

A number of methods exist for detecting gene regulatory proteins in crude extracts. Most of these have been tested for their effectiveness in prokaryotic systems. In table 1 a list of the major methods is given and in tables 2 and 3 the methods actually used for monitoring the isolations for a number of prokaryotic and eukaryotic proteins respectively are given. The amount of regulatory protein in a crude unfractionated extract is often very small and so the existence of an assay that can be used to detect the regulatory protein under such conditions is crucial for monitoring purification. There



**Table 1.** The main methods available for detection of gene regulatory proteins in cell extracts.

Assay	Comments
1. Affinity for small molecule effector.	Usually limited by lack of sufficient specificity and low affinity. Used for isolation of lac repressor routinely used for steroid hormone receptors.
2. DNA-binding assay.	Potentially very useful because of high affinity between regulatory protein and target site on DNA. Possible interference by other proteins in crude extract. Largely untested as an assay tool for regulatory proteins in crude extracts.
3. Selective effect on DNA-directed transcription (in prokaryotes or eukaryotes) and/or translation (prokaryotes only). In the latter case it is known as the coupled system assay.	The most commonly used assay for prokaryotes combines sensitivity with selectivity. Applications to eukaryotes have led to a few successes and a number of frustrations.
4. Affinity chromatography.	Applicable where one of the components that interacts with the regulatory protein can be purified and covalently attached to a column. The method is useful both for detection and purification.
5. Construction of an overproducer strain.	This requires that the regulatory gene be available. Typically the gene in question is linked to a multicopy plasmid with a strong promoter so that the protein in question can be readily detected by SDS-polyacrylamide gel electrophoresis or a suitably induced extract. Potentially applicable to eukaryotes in which the cDNA copy of the regulatory gene is available.

are two paramount considerations in choosing an assay procedure: sensitivity and selectivity (or specificity). Other factors being equal one would undoubtedly choose the most convenient procedure.

The sensitivity of an assay is directly related to the affinity between the regulatory protein and the probe used for its detection. In fact the concentration of regulatory protein that can be detected with a given probe is approximately equal to the dissociation constant between the regulatory protein and the probe. For example, in the case of the lac operon, the dissociation constant,  $K_D$ , for the repressor-IPTG complex is about  $10^{-6}$  M whereas the  $K_D$  for the repressor-operator complex is about  $10^{-10}$  M. Clearly a much lower concentration of lac repressor would be required for an assay involving the binding of repressor to the appropriate DNA. The coupled system could also be used to assay repressor. In this case one would measure repressor as a function of its inhibiting effect on  $\beta$ -galactosidase synthesis. Since the assay in effect is a measure of repressor-operator complex formation it would be expected to have the same sensitivity as the direct DNA-binding assay.

The selectivity of an assay is not simply related to any one factor in the system. Rather it is related to the extent to which the desired result can be observed above side reactions that might result between the probe and the various factors present in the crude extract.

**Table 2.** Some gene regulatory proteins that have been isolated and characterized from prokaryotes.

Protein	Function	Assay used to monitor Isolation
lac repressor	Represses lac operon.	Anti repressor binding
ara C regulatory protein	Functions in different forms as a repressor and an activator of the arabinose operon	Coupled system
arg R repressor	Represses transcription of genes involved in arginine biosynthesis	Coupled system
trp R repressor	Represses transcription of the trp operon	Coupled system
CAP	Activates catabolite gene transcription in the presence of cAMP	Coupled system
gal R repressor	Represses transcription of the gal operon	Coupled system
$\lambda C_1$	Activates and represses transcription of the $C_1$ gene at different levels; represses early right and early left transcription.	Direct detection by selective labelling in an overproducer strain.
$\lambda cro$	Represses $\lambda C_1$ transcription at low concentrations. Also represses early right $\lambda$ transcription at high concentrations.	Direct detection in a plasmid over-producer strain.
$\lambda N$	Anti terminator leading to late $\lambda$ transcription	Coupled system
lex	Represses transcription of genes involved in DNA repair.	Direct detection in a plasmid over-producer strain.
$\lambda Q$	Antiterminator leading to late $\lambda$ transcription	Direct detection in a plasmid over-producer strain.

**Table 3.** Some gene regulatory proteins that have been isolated and characterized from eukaryotes.

Protein	Function	Assay used to monitor purification
40 kd Xenopus protein	Activation of Xenopus 5S gene transcription	Transcription
SV40 T antigen	Repressor of early SV40 transcription	Precipitation by selective antiserum isolated from tumor producing animals
Sp1 protein	Stimulates early SV40 transcription presumed to function in same capacity for unknown host genes.	Transcription assay
Glucocorticoid receptor	Activates transcription of glucocorticoid responsive genes	Steroid hormone binding

In the case of the assay for lac repressor it was stated that the DNA binding as the coupled system assay would be expected to have about the same sensitivity. This is not true for the selectivity of the two assays. First, consider the problem of the DNA-binding assay to detect the lac repressor in a crude extract. The lac repressor is present at very low concentration in a crude extract. Addition of labelled lac operator containing DNA to such a crude extract would be expected to lead to specific complex formation and consequent retention of a significant percentage of the DNA on a nitrocellulose filter as long as the concentration of repressor was adequate, i.e.,  $10^{-8}$  M or greater. However, many other proteins with a potential for binding to DNA are present in the crude extract so that non specific retention could make it difficult to detect specific binding by this method. The problem would be expected to be more serious the larger the DNAs since this makes more nonspecific sites available for binding. The selectivity problem could be further compounded if the repressor was not completely specific for binding to the lac operator. In fact this is not a problem for lac repressor but it is for other gene regulatory protein such as CAP (Guthrie *et al.*, 1975) and glucocorticoid receptor discussed above. No further comments on this approach can be made since lac repressor-DNA binding, like most other DNA-protein complexes, has only been studied with purified repressor or highly enriched extracts. Now consider the advantages of the coupled system. In this case the assay is based on the selective inhibition of lac mRNA or  $\beta$ -galactosidase synthesis that is reversed in the presence of antirepressor IPTG. It seems likely that the only factor in a crude extract that could produce such an inhibitory effect would be the lac repressor itself. Such an assay, therefore, would be expected to have near absolute specificity and would obviously be preferable to the DNA-binding assay when it is available.

It is principally of historical interest that the lac repressor was isolated using a DNA binding assay to radioactive antirepressor, [ $^{14}\text{C}$ ]-IPTG (Gilbert and Muller, 1966). Because of the large dissociation constant of this complex it was possible to isolate the repressor only after the construction of a mutant strain that produced more than the normal amount of repressor. Clearly other assay procedures would have been preferable if they were not in wide use at the time. Subsequently the coupled system assay was used to isolate CAP activator protein by its specific cAMP-dependent stimulation of  $\beta$ -galactosidase synthesis. Similarly the coupled system approach was used to isolate a number of other regulatory proteins that bind to DNA or RNA polymerase; these included the ara C activator/repressor, the arg R repressor, the trp R repressor, the lac R repressor and the  $\lambda$  N antiterminator.

Thus the coupled system approach has established itself as a popular means of assaying for regulatory proteins. It should be emphasized that this is only applicable to prokaryotes where coupling of transcription and translation is a natural process.

Sometimes the regulatory gene in question can be made to overexpress to the point where its presence in a crude extract can be directly detected by protein gel analysis. Use of this approach usually presupposes the availability of the regulatory gene for purposes of manipulation. The gene can be attached to a multicopy plasmid together with a highly active promoter so that several per cent of a suitably transfected and induced *E. coli* cell should be that protein. This should lead to a prominent band on gel that could be used to monitor the purification. Such a cell source obviously also would facilitate purification because of the higher concentration of the desired protein.

in the crude extract. This is clearly a very attractive approach with the one major limitation of requiring that the regulatory gene (or its cDNA copy in the case of a eukaryotic gene with introns) be available.

Transcriptional assays, where the immediate RNA product of this gene is analyzed, are increasing in popularity. Particularly in eukaryotes where transcription and translation are physically separated processes. In theory the direct transcription assay should be highly selective and sensitive provided the transcript can be adequately characterized. Frequently the 'runoff assay' in which the fragmented gene is used as template is a convenient device for running the transcription assay. In this case the promoter proximal fragment, appropriately labelled, is identified by autoradiography after gel electrophoresis (Manley, 1983). Remarkable progress has been made with *in vitro* transcription systems using extracts from various eukaryotes. However, this progress has been limited to elucidation of certain basic aspects of transcription such as determining correct initiation sites, capping and splicing. Relatively little progress has been made in studying regulatory aspects of transcription in such systems. Why this is so is unclear. But it may be symptomatic that the importance of the enhancer element to transcription is not reflected in the cell-free systems used to study transcription. Perhaps the nuclear architecture or other subtle features of the *in vitro* situation are not reflected by the *in vitro* systems currently in use. Whatever these problems may be their solution is of paramount importance since it is clear that transcription systems will be a most useful tool both to assay regulatory factors in crude extracts and to characterize them once they are purified.

Despite these apparent problems two notable achievements have been made with the transcription system assay. These involve the work on the 40kd regulatory protein required for activation of the 5S genes in *Xenopus* and the isolation of the Sp1 factor required for early transcription of SV40.

It should also be noted that the SV40 T antigen could probably have been isolated with the help of a transcription assay. In fact it was isolated initially with the help of an antiserum assay.

The most successful approach to isolating eukaryotic gene regulatory proteins has been in the area of steroid hormone receptor proteins. This has been possible because of the relatively high affinities between the small molecule effector (the steroid hormone) and the regulatory protein (steroid receptor protein). The Kds for these complexes tend to be smaller than  $10^{-8}$  M. The greatest disappointment with these steroid receptor complexes is that it has not been possible to demonstrate any effect *in vitro* on transcription. This could be due to some damage to the complex on isolation or to other reasons referred to above.

In conclusion it should be emphasized that the search for regulatory proteins in eukaryotes is in its infancy. A great deal of methodology can be borrowed from the successes with prokaryotes; a great deal of new methodology will undoubtedly have to be developed. There are serious handicaps which have to be overcome. These include mutants in regulatory proteins of interest, the isolation of regulatory genes and *in vitro* transcription systems which are sensitive to the effects of regulatory proteins. In the midst of all these limitations it seems reasonable that more effort should be directed towards exploiting the DNA binding assay. As pointed out DNA-binding of regulatory proteins has been studied almost exclusively with purified proteins. The strength of

interaction shown by such studies is high encouraging the belief that this assay has adequate sensitivity for detection of specific regulatory proteins in crude extracts. The big question is whether experimental conditions can be manipulated so that the DN-binding assay will show sufficient selectivity to be used in conjunction with crude extracts.

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## Genetic transformation in bacteria

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**Abstract.** Certain species of bacteria can become competent to take up high molecular weight DNA from the surrounding medium. DNA homologous to resident chromosomal DNA is transported, processed and recombined with the resident DNA. There are some variations in steps leading to transformation between Gram-positive bacteria like *Diplococcus pneumoniae* and Gram-negative bacteria represented by *Haemophilus influenzae* but the integration is by single-strand displacement in both cases. Plasmid (RSF0885) transformation is low in *Haemophilus influenzae* but this is increased significantly if (homologous) chromosomal DNA is spliced to plasmid DNA. In *Haemophilus influenzae*, *rec1* function is required for peak transformation with chimeric plasmids. Chimeric plasmid fixed presumably extrachromosomally undergoes frequent recombination between homologous segments contained in resident chromosome and the plasmid.

**Keywords.** DNA-cell interaction; competence; specificity in DNA uptake; single-strand displacement; chimeric plasmid transformation; *rec1* function; recombination.

### Introduction

In the state of competence, certain species of bacteria, notably *Streptococcus pneumoniae*, *Bacillus subtilis* and *Haemophilus influenzae* can take up high molecular weight ( $M_v$ ) DNA from the surrounding medium. The end result of this interaction depends on whether the input DNA is homologous or heterologous, that of a phage or of a plasmid. In the event of input DNA being homologous, the resident DNA abstracts the information contained in the input DNA by a physical recombinational mechanism. Thus, the recipient cell would be heritably altered or transformed for one or more incoming gene markers. Phage DNA introduced in a similar manner produces phage particles although the efficiency of transfection is much lower than that of infection. Finally, the input plasmid DNA is fixed extrachromosomally, thereby 'adding' markers to the cell. At least in certain cases, the efficiency of transformation of a plasmid marker is lower than that for a homologous DNA marker. Chimeric plasmids have certain additional requirements in some systems which will also be discussed. More detailed reviews have appeared earlier (Spizizen *et al.*, 1966; Tomasz, 1969; Notani and Setlow, 1974; Lacks, 1977; Smith *et al.*, 1981; Goodgal, 1982).

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Abbreviations used:  $M_v$ , Molecular weight; CF, competence factor; BF, binding factor.

### Introduction of competence

Methods to induce very high levels of competence in *S. pneumoniae* and *H. influenzae* have been developed. A pneumococcal culture becomes competent as it reaches certain cell density ( $10^7$ – $10^8$  cells/ml) during exponential phase growth but this state lasts for about 15 min or so. Competence in pneumococcus is initiated by competence factor (CF) of 5–10 kdal. It is made by the cell and is excreted in the medium. Exogenous CF will also induce competence. Two phases have been discerned (Ziegler and Tomasz 1970). In the first phase CF interacts with the cell receptor (protein) to form a complex. In the second phase incompetent cell (CF) requires new protein synthesis to yield a competent cell culture (Tomasz, 1970). The new protein synthesis may include induction of an autolysin whose limited action on the cell wall may unmask the DNA binding sites (Seto and Tomasz, 1974). A DNA binding factor (BF) has been selectively released which has the properties of a DNA receptor (Seto and Tomasz, 1975). Lacks and Neuberger (1975) have reported the membrane localization of a deoxyribonuclease which is involved in transformation (Lacks, 1962; Lacks et al., 1974). Apparent autolysin exposes both DNA BF and the DNase for DNA uptake and entry.

Morrison and Baker (1979) labelled proteins synthesized before and during competence. They noted a dramatic decrease in most of the precompetence proteins. During competence only 11 major species of proteins were labelled, out of which only 19.5 kdal species appeared definitely new although there was a hint of 3 others. If CF was inactivated these bands did not appear. Surprisingly, the main competence-specific 19.5 kdal protein band was found similar in its electrophoretic properties to purE eclipse complex protein (see later). In *S. sanguis*, Raina and Ravin (1980) also observed synthesis of a set of 10 proteins, 8 of which were early and 2 were late. One of the early proteins, E16, with  $M_r$  of 15500 binds specifically to donor DNA after uptake. It was presumed that this protein is involved in translocation of DNA from cell membrane to the chromosome.

In contrast to pneumococci, *B. subtilis* cultures develop a relatively low level of competence—upto 18% of the cells become competent (Bott and Wilson, 1968). It requires almost 8 h of growth in the synthetic medium of which 2–3 h are after cessation of exponential growth. Singh and Pitale (1968) could enrich competent cell fraction by sedimenting through sucrose gradients. Competent cells sediment slower than non-competent cells. Similarly, Cahn and Fox (1968) could separate competent from noncompetent cells by the differences in their buoyant densities in a Renografin gradient. Lighter density cells were the competent fraction and these constituted upto 10% of the total.

*H. influenzae* and *H. parainfluenzae* appear to become competent when the cell division is blocked but protein synthesis is allowed to take place. No CF appears to be involved (Herriott et al., 1970). A number of *H. influenzae* *com*<sup>-</sup> (competence-defective) mutants have been isolated (Caster et al., 1970; Postel and Goodgal, 1972). Kahn et al. (1979) reported DNA binding activity of vesicles produced by competence-deficient mutants of *Haemophilus*. A number of polypeptides, presumably involved in competence development or DNA uptake, were detected by Concino and Goodgal (1981) but none of these were specifically implicated in a competence function. On the other hand, Concino and Goodgal (1982) observed that *com* 51 released vesicles containing

DNA receptor proteins during competence development which had the capacity to bind homologous but not heterologous DNA. Kahn *et al.* (1982) observed morphological differences between competent and non-competent cells of both *H. influenzae* and *H. parainfluenzae*. When thin sections were examined by electron microscopy they noted that competent *H. parainfluenzae* had five times as many membranous extensions as non-competent cells. Moreover, upon exposure to transforming DNA, *H. parainfluenzae* cell extensions disappeared and were apparently internalized. The name 'transformasome' has been proposed for the membranous extensions developed during competence of *Haemophilus* cells which apparently are involved not only in the uptake but also for protecting DNA even when unmethylated from restriction nuclease attack (Kahn *et al.*, 1983). An odd observation is that *Haemophilus* transfection with phage DNA is efficient only with cells made competent by aerobic-anaerobic-aerobic rather than MIV method.

*Neisseria gonorrhoeae* is another Gram-negative bacterium that is transformable and surprisingly throughout its growth phase. Virulent colony types are transformable with a high frequency (upto 1 %) and non-virulent with very low frequency ( $10^{-7}$ ). Virulent colony types are also piliated and non-virulent ones are not and thus are correlated with competence. There is no evidence for other competence factors (Biswas *et al.*, 1977).

### Specificity in DNA for uptake

While competent pneumococcal cells can take up virtually any high  $M_r$  DNA, there seems to be some specificity involved in *H. influenzae*. Thus, Scoocca *et al.* (1974) observed that *H. influenzae* can take up its own DNA with a remarkable efficiency but not that of *Escherichia coli* B or *Xenopus laevis*. Only *H. parainfluenzae* DNA could compete with *H. influenzae* DNA. The basis for this specificity has been shown to be an uptake site (recognition sequence) in DNA, (Sisco and Smith, 1979). Deich and Smith (1980) have inferred that regardless of the input DNA length (from 1–40 kb), the efficiency of uptake binding sites is the same. Danner *et al.* (1980) sequenced 4 small fragments of *H. parainfluenzae* to find the common sequence in the ones that are not taken up efficiently and its absence in the ones that are taken up poorly. They came up with an asymmetric 11 bp sequence 5' AAGTGC GGTC A 3' as the uptake sequence. They also showed that ethylation of phosphoryl groups in this sequence reduced uptake significantly. Danner *et al.* (1982) synthesized the 11 bp uptake sequence and constructed a series of plasmids incorporating this sequence. They concluded that this sequence is necessary and sufficient for uptake in *H. influenzae*. However, there could be as much as 48-fold difference in uptake activity and this they attributed to AT-richness of the DNA next to the sequence. Using sonicated DNA, Vogt and Goodgal (1984a) inferred that the number of uptake signals has been underestimated before and this discrepancy was accounted for by assuming multiple receptors which bind to a high  $M_r$  DNA containing multiple uptake signals. Vogt and Goodgal (1984b) also suggested that the uptake recognition signal may have its basis in 'structural capacity' which may be non-specifically dependant on a sequence.



### The nature of irreversibly-bound intracellular input DNA

The initial binding to a competent cell may be reversible by external agents but very soon it is not removable by DNAase. The irreversibly-bound DNA in pneumococcus is not recoverable in native form—part of it is degraded and part of it is denatured (single stranded). Entry of DNA into pneumococcus appears to be coupled with its becoming single-stranded with concomitant degradation of the complementary strand (Lacks 1962; Lacks *et al.*, 1974). Surprisingly, a double mutant with two deficient DNases was normal for entry and post-uptake events. However, further selections made for high deficiencies of the nucleases and those deficient in transformation provided illuminating information. Two classes of transformation-deficient mutants were isolated. One which did not bind DNA (*ntr* mutants) and the other bound and accumulated DNA on the surface but the DNA did not enter. These latter cells were missing a DNase (*n* mutants). A DNA translocase function has been postulated for this enzyme (Lacks *et al.*, 1974). *noz* mutants are probably additional mutation(s) in the *end* gene. Lacks and Neuberger (1975) have localized endonuclease I in the membrane which appears to be responsible for converting double-stranded DNA to single-strand inside and oligonucleotides outside. They also localized autolysin in the membrane. BF and *end* I may be exposed by the action of autolysin. *ntr* mutants may lack BF.

Intracellular single-stranded pneumococcal DNA is in an 'eclipse' i.e. is not biologically active. It is 'coated' by a 19.5 kdal protein. Recovery from eclipse occurs following integration. The role of eclipse protein may be to promote its entry into the cell, to protect it and to have it in an 'integrational' conformation (Morrison, 1977).

In *B. subtilis*, Bodmer and Ganesan (1964) found intracellular input DNA in the native, hybrid and denatured form. Eisenstadt *et al.* (1975) reported a denatured DNA binding protein activity from *B. subtilis* competent cells. This protein protects denatured DNA from nuclease solubilization. This activity may be analogous to pneumococcal eclipse protein or may have some other function.

Unlike in pneumococcal or *B. subtilis* transformation, no denatured input DNA is detected intracellularly in *H. influenzae* (Notani and Goodgal, 1966). As a matter of fact the bulk of unintegrated DNA appears to be in a native form and is biologically active (Notani, 1971). However, slower-sedimenting fragments with lower biological activity, designated species II molecules, are observed but these were considered not intermediates but byproducts of transformation (Notani, 1971). Two of the recombination deficient mutants, *rec* 1 and *rec* 2, have been analyzed. With *rec* 2, DNA is irreversibly bound but is reisolatable without appreciable change. DNA is neither degraded nor is it integrated. On the other hand, with *rec* 1 the radioactive label in the input DNA is transferred to resident DNA but genetic information is not transferred (Notani *et al.* 1972). Transformation in *H. parainfluenzae* also yields intracellular DNA in a biologically active form and is not denatured (Notani and Setlow, 1972). *Haemophilus* phage 1 (HP1) DNA introduced by transfection also undergoes fragmentation. However, only in recombination-proficient strain part of it is reassembled yielding some phage biological activity (Notani *et al.*, 1973). Double-stranded nature of unintegrated donor DNA is a bit surprising because the integration appears to be primarily single-stranded (Notani and Goodgal, 1966). Elsewhere it is discussed that *Haemophilus* DNA first enters into transformosome and from there linear DNA exits.

into the cell more rapidly than the covalently-closed circular DNA. It may be conjectured that *rec 2* mutant has its exit pore blocked or that some nuclease is missing so that the initial processing of DNA does not occur.

### Genetic recombination and integration of chromosomal DNA

Using radioactivity and density labels, Fox and Allen (1964), Fox (1966) showed that newly introduced DNA is a hybrid and physically a heteroduplex. The size of insert is about 1–2 million daltons. The picture of integration from *H. influenzae* is somewhat similar, even though a free single-stranded intermediate is not observed. The kinetics of integration could be followed even without a density label in the donor DNA. Bulk separation of donor DNA from resident DNA + integrated DNA was achieved by zone sedimentation (Notani and Goodgal, 1965; Notani *et al.*, 1966). However, intact physical integration of donor DNA could not be shown from those experiments. By density-label experiments, it was observed that in *H. influenzae* transformation also single-stranded donor DNA segments are integrated into the genome (Notani and Goodgal, 1966). The average size of the integrated segments was estimated at  $5\text{--}6 \times 10^6 M_r$ . Subsequently, it was also shown that either strand is effective in transformation (Goodgal and Notani, 1968). In *B. subtilis* transformation, donor density-label atoms were found in native recipient, hybrid and 'denatured' donor DNA (Bodmer and Ganesan, 1964). There is evidence for single-stranded integration (Bodmer, 1965). It has been shown also for *B. subtilis* that transforming activity resides in both complementary strands (Chilton, 1967).

Genetic recombination during transformation occurs apparently by a single-strand invasion and displacement (Fox, 1966). In *H. influenzae* also a similar model would be valid with the proviso that the initial interaction takes place between two double-stranded DNA molecules out of which one-strand is integrated and the other degraded (Notani and Goodgal, 1966) and the interaction would produce a byproduct of species II molecules (Notani, 1971). There is little information on the proteins that are involved except the circumstantial evidence that *H. influenzae rec 1* gene may be a type of annealing protein (Notani *et al.*, 1973).

The primary product during transformation is a duplex which is heterozygous for the input and resident gene marker. A mismatch at the site of heterozygosity would thus be expected. In pneumococcus, it has been observed depending on their relative efficiency of transformation of single-site markers can be grouped into four categories *viz.* VHE, HE, IE and LE with relative efficiency ratios of 1:0.5:0.2:0.05 respectively (Lacks, 1966). LE markers are low in efficiency presumably because donor DNA strand is corrected out more often. In  $\text{Hex}^-$  mutant cells, transformation of LE marker can be raised to that of HE. For amylomaltase *mal P* gene, Lacks *et al.* (1982) observed that transition mismatches are highly corrected by repair but transversion mismatches are less susceptible. With LE markers the donor DNA is apparently recognized as 'foreign' and may be attracting restriction mechanisms. Claverys *et al.* (1983) looked at the mismatch repair events at *ami A* locus. They observed that LE markers came from transitional changes  $\text{AT} \rightarrow \text{GC}$ . IE markers arise from transversional changes  $\text{AT} \rightarrow \text{TA}$  and HE from transversions  $\text{GC} \rightarrow \text{TA}$  or  $\text{GC} \rightarrow \text{CG}$ .

### Transformation with plasmid and chimeric DNA

Plasmid RSF0885 a multicopy plasmid originally isolated from *H. influenzae* b h been extensively used in transformation and recombinant DNA work in *H. influenzae* Rd. It has a  $M_r$  of  $3.7 \times 10^6$ , carries an *amp<sup>r</sup>* marker and is cut once with *Pvu* II outside the marker. Since *H. influenzae* cells become almost 100% competent, it was expected that RSF0885 should give high transformation. As a matter of fact, only about 10 transformants/ $\mu$ g of DNA were obtained which was 3-4 orders of magnitude lower than expected (Notani et al., 1981). Transformation with RSF0885 did not require the expression of *rec 1* or *rec 2* genes. It was observed also that chromosomal DNA effectively competes with the plasmid DNA and essentially wipes out the plasmid transformation. These observations were interpreted in the light of reports of (i) Scofield et al. (1974) that *H. influenzae* binds its own DNA well but not that of *E. coli* B and suggested that there is some specificity in uptake of DNA by *H. influenzae* and (ii) Sisco and Smith (1979) and Chung and Goodgal (1979) that *H. influenzae* DNA contains recognition regions (uptake sites which are responsible for the specificity of uptake). Sisco and Smith (1979) noted that 600 copies of an 11 bp sequence are distributed in the *H. influenzae* genome. The low efficiency of transformation with RSF0885 was thus attributed to the plasmid DNA not containing any uptake sequence.

Consistent with this notion it was observed that when chromosomal DNA was spliced to plasmid DNA the transformation for the plasmid marker increased 2 to 3 orders of magnitude (Notani, 1981; Setlow et al., 1981). Surprisingly for efficient chimeric plasmid transformation, both *rec 1* and *rec 2* gene expression was required. Both *rec 1* and *rec 2* are deficient in recombination but the exact role of recombination in chimeric plasmid DNA transformation is not established.

Setlow et al. (1981) cloned a *nov<sup>r</sup>* (25  $\mu$ g/ml level) marker. When this chimeric plasmid pNov 1 was used in transformation, 100-fold more *nov<sup>r</sup>* than *amp<sup>r</sup>* transformants were observed. None of the novobiocin transformants (17 examined) contained the plasmid but only 5 out of 20 *amp<sup>r</sup>* transformants contained *nov<sup>r</sup>* marker but plasmid was present in all the cases and was of the same size. Joshi and Notani (1983) have constructed another vector pJ1-8 which was derived from pD7 (Notani, 1981) consisting of RSF0885 + unmarked chromosomal DNA insert. pJ1-8 has a  $M_r$  of only  $2.5 \times 10^6$  and is cut once with *Eco*RI. It gives virtually no transformation for *amp<sup>r</sup>*. Splicing chromosomal DNA to pJ1-8 increases the *amp<sup>r</sup>* transformants significantly providing even a more facile assay than with RSF0885. Using this plasmid several *nov<sup>r</sup>* (2.5  $\mu$ g/ml level) clones were isolated. Three of these were characterized in detail. In general, observations are in agreement with those of Setlow et al. (1981). Some additional observations have been made.

When three different clones containing *nov<sup>r</sup>* (2.5) marker were used in transformation, transformation was differential and the ratios of *amp<sup>r</sup>:amp<sup>r</sup>nov<sup>r</sup>* were somewhat different. Whereas pJ1-8N2 and pJ1-8N20 yielded *amp<sup>r</sup>:amp<sup>r</sup>nov<sup>r</sup>* ratios of 2:1, pJ1-8N19 gave a ratio of up to 6:1 implying that more recombinants (*amp<sup>r</sup>nov<sup>r</sup>*) were generated with pJ1-8N19 (Joshi, V. P. and Notani, N. K., unpublished observations). Balganesch and Setlow (1984) have also observed different recombination percentages with different clones but they have correlated this with the length of the insert.

A number of chimeric plasmids in *H. influenzae* have been found to be unstable.

Setlow *et al.* (1984) have observed that such plasmids are however stable in a strain which lacks or does not have an inducible phage.

Kahn *et al.* (1983) and Barany *et al.* (1983) have inferred that *H. influenzae* DNA following uptake enters into membranous extensions called transformasomes and DNA there is protected against external nucleases and internal restriction enzymes. Entry into the cell apparently is much faster if the DNA is linear but slower if circular. These experiments were done with plasmids, pCML6 or pPUP3, both of which utilize pBR322 replicator and the only *H. influenzae* DNA that pPUP3 has is the 11-bp uptake sequence. It is not known if pBR322 can replicate in *H. influenzae* and whether that would contribute to any of the observed effects. Furthermore, there is a requirement of *rec* gene expression for transformation with chimeric plasmids which in the case of pPUP3 may have created a problem.

In reextraction experiments in which a chimeric plasmid pD7 (RSF0885 replicator + chromosomal DNA) was used and reisolated plasmid DNA analyzed by sucrose sedimentation, it was observed that not only the relative specific biological activity goes down by about 60% in 60 min but that profile of the plasmid transforming activity also changes. In 60 min profiles although the activity is somewhat dispersed more, the peak biological activity is surprisingly obtained from fractions sedimenting faster than at 0 min time. This indicates that in 60 min time more than 60% of the material is processed or inactivated. In *S. pneumoniae*, a multicopy plasmid pMV158 has been utilized to clone genes. A gene for maltose was successfully cloned and expressed in strains having a deletion of the locus (Stassi *et al.*, 1982). Lopez *et al.* (1982) have made a distinction between *plasmid transfer* and *plasmid transformation*. In the former case, the recombinant plasmid is established by itself and in the latter case an endogenous homologous plasmid is altered. *Mal*<sup>+</sup> transformant frequency was only slightly higher ( $5 \times 10^4$ ) by transformation (*mal* deletion in chromosome and pMV158) than by transfer ( $1.3 \times 10^4$ ) i.e. with only *mal* deletion in the chromosome. Since entry of DNA in *S. pneumoniae* is by a denaturation and degradative process, authors have proposed models which allow interaction between DNA molecules or with itself to yield intact plasmids.

In *S. pneumoniae*, Barany and Tomasz (1980) reported transformation for drug resistance markers with several heterologous plasmid DNAs. The frequencies ranged from  $5 \times 10^{-1}$  to  $10^{-5}$ . This transformation did not occur with an endonuclease I deficient strain. Unlike in *B. subtilis* in which competent cells are transformed mainly by oligomers, in *S. pneumoniae* monomers are quite effective.

In *B. subtilis*, Canosi *et al.* (1978) and Mottes *et al.* (1979) showed that transformation in *B. subtilis* of competent cells is mainly by oligomers. However, *B. subtilis* protoplasts can be transformed by monomers and with a very high efficiency. Approximately  $4 \times 10^7$  transformants were obtained per  $\mu\text{g}$  of CCCDNA (Chang and Cohen, 1979). Recombinant plasmids could also transform although with a lower efficiency (one to three orders of magnitude lower). Canosi *et al.* (1981) reported that monomeric form of pC194 transforms efficiently if chromosomal DNA is spliced to it. These observations have been interpreted by Canosi *et al.* (1981) to suggest the operation of two pathways: a major one requiring *rec* gene expression and homology between recipient and donor DNAs and a minor one *rec*-independent and homology-independent. The processing of incoming plasmid DNA requires conversion of single-stranded, denatured form

observed by deVos *et al.* (1981) to a double-stranded circular active form through *rec E4* gene expression. The exact physiological role of *rec E4* gene is unclear at present.

## Discussion

Genetic transformation in bacteria has revealed several unusual features which include interaction between cell (receptors etc.) and DNA, the transport of the latter across the cell envelope, protection and preparation of DNA for recombination, the interaction between two DNA molecules and finally the act of recombination itself. Gram-positive (namely pneumococci) and Gram-negative (namely haemophilus) bacteria seem to have developed somewhat different mechanisms although the end result is homologous displacement of resident DNA segments by input (chromosomal) DNA. While pneumococci use the CF, autolysin, BF, an endonuclease and 'eclipse' protein to do the uptake, protection and transport of DNA, *Haemophilus* shows a specificity in DNA uptake. Sequestering of DNA irreversibly-bound in transformasomes presumably to protect DNA from restriction is also a novel feature of this system. Also, whereas intracellular transforming DNA in pneumococci is recoverable only as degraded or denatured (single-stranded) material, bulk of intracellular donor DNA in *Haemophilus* is recoverable in native form. Of course some amount of degradation and fragmentation is also observed.

The role of *rec* genes in promoting transformation with chimeric plasmids is not readily understood. It may be conceivable that after pairing, resident chromosomal DNA may physically assist its entry into the cell. We have observed that recombination with certain chimeric plasmids takes place quite early. In addition, majority of the fixed plasmids are recombinant for the marked region. We have imagined that the fixation of a recombinant plasmid is preceded by resident DNA single-strand invasion of the plasmid, followed by mismatch correction in which the resident marker would be eliminated 50% or more than 50% of the time.

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## Receptor-mediated endocytosis: An overview of a dynamic process

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**Abstract.** The decade of the 70's was remarkable for the insights that rapidly accumulated to provide us with an understanding of one of the fundamental processes of animal cell metabolism, namely, how mammalian cells ingest a host of extracellular substances to satisfy their various metabolic needs. It has long been appreciated that the surfaces of mammalian cells are in a continual state of flux. Surface membranes often fold inward and pinch off in a vesicular form trapping some of the contents of the extracellular material which are thus transported into the cell. This process is called endocytosis (reviewed in Silverstein *et al.*, 1977). When extracellular fluids are taken up in this manner, the process is called fluid-phase endocytosis or pinocytosis. When solids are ingested, the process is called phagocytosis. Although quantitatively important over the long run, these modes of uptake are slow, non-specific and dependent on the concentration of the substance in the extracellular medium. In recent years it has been recognized that animal cells have developed a specialized form of this vesicular transport system to selectively retrieve and assimilate macromolecules from the extracellular milieu with high efficiency. This process is called receptor-mediated endocytosis. In this review an attempt is made to collate and correlate the evidence establishing receptor-mediated endocytosis as a dynamic process that routes cell surface receptors and ligands through multiple intracellular compartments to their ultimate destination.

**Keywords.** Endocytosis; low density lipoprotein; receptor recycling; lysosomotropic agents; ionophores.

### Pathway for receptor-mediated endocytosis

The key to the efficiency of the process of receptor-mediated endocytosis is the existence of specific cell surface receptors which recognize and bind specific macromolecules to the cell surface with high affinity. Such high affinity receptors permit selective retrieval of specific macromolecules present in relatively low concentrations in the extracellular fluid. Following binding to the receptors the ligands are internalized with remarkable efficiency through the operation of a vesicular transport system the outline of which first became apparent from the studies of Goldstein, Brown and their colleagues on the uptake of LDL by cultured human fibroblasts (reviewed in Goldstein *et al.*, 1979, 1983).

Low density lipoprotein (LDL) is the major cholesterol-carrying protein in human plasma. It is a large spherical particle (molecular weight,  $M_r \sim 3 \times 10^6$ , 220 Å

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Abbreviations used: LDL, Low density lipoprotein; EGF, epidermal growth factor; CURL, compartment of uncoupling of receptor and ligand,  $M_r$ , molecular weight; CoA, coenzyme A.



diameter) with an apolar core composed primarily of cholesterol esters. This apolar core is enclosed in a polar coat composed of phospholipids, a small amount of free cholesterol and a single large protein called apoprotein B. Despite their capability for *de novo* cholesterol biosynthesis, most mammalian cells preferentially utilize cholesterol carried in the LDL particles as cholesterol esters. The sequence of reactions involved in the utilization of the LDL-derived cholesterol by mammalian cells is called the LDL receptor pathway.

The initial reaction in the LDL receptor pathway is the binding of LDL to specific high affinity cell surface receptors (Goldstein *et al.*, 1974, 1976). The binding requires divalent cations,  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ , and is specific for the apoprotein B moiety of LDL. The LDL receptors on the fibroblast cell surface appear to be similar to those on bovine adrenal cortex cells (Basu *et al.*, 1978; Kovanen *et al.*, 1979). The LDL receptor has been solubilized from bovine adrenal cortex and purified to homogeneity (Schneider *et al.*, 1979, 1982). It is a transmembrane glycoprotein ( $M_r$  164,000). In the cultured fibroblasts it is synthesized in a precursor form ( $M_r$  120,000) which undergoes posttranslational glycosylation presumably in the Golgi apparatus to the mature form ( $M_r$  164,000) (Tolleshaug *et al.*, 1982).

Early kinetic experiments using [ $^{125}\text{I}$ ]-LDL established that the receptor-bound LDL is rapidly internalized (half-time for internalization: 5 min) and eventually delivered to the lysosomes where its protein and cholesterol ester components are hydrolyzed (Goldstein *et al.*, 1974, 1976). Three regulatory responses ensued in the cell following the lysosomal degradation of the LDL (Brown and Goldstein, 1975). *De novo* cholesterol synthesis was inhibited through suppression of HMG coenzyme A (CoA) reductase (mevalonate: NADP $^+$  oxidoreductase (CoA-acylating), EC 1.1.1.34). Exogenous free cholesterol was reesterified through stimulation of microsomal acylcoenzyme A:cholesterol acyl transferase (acyltransferase, EC 2.3.1.26). Finally, the synthesis of surface LDL receptors was suppressed precluding an inordinate uptake of LDL. Inasmuch as the rate of internalization of the receptor-bound LDL was much faster than the overall turnover rate of plasma membrane components, these kinetic studies suggested that the distribution of the LDL receptors on the cell surface was not random (Goldstein *et al.*, 1976).

Direct corroboration of these biochemical experiments was adduced by Anderson *et al.* (1976, 1977a) who used LDL labelled with the electron-opaque probe ferritin in electron microscopic kinetic studies. They found that in normal fibroblasts LDL-ferritin was localized in specialized regions of the cell surface where the plasma membrane was indented and coated on the cytoplasmic side by a fuzzy material. These regions called 'coated pits' occupy only about 2% of the surface area of fibroblasts. Within 50–80% of the LDL-ferritin was bound in these regions. Within a minute of binding of LDL-ferritin the coated pits invaginated into the cells. Within 3 min the LDL-ferritin was found in intracellular coated vesicles. These vesicles rapidly lost their coats and fused with other intracellular vesicles including lysosomes within about 5 min.

The LDL receptor pathway seems to be a prototype for receptor-mediated endocytosis of macromolecules. Similar mechanisms mediate the uptake of a number of transmembrane proteins (transferrin, transcobalamin II), polypeptide hormones [insulin, epidermal growth factor (EGF)], glycoproteins (asialoglycoproteins, lysosomal enzymes), plasma proteins ( $\alpha_2$ -macroglobulin, maternal immunoglobulins), immune complexes, vir-

(Semliki Forest virus) and toxins (diphtheria toxin, *Pseudomonas* toxin) (reviewed in Kaplan, 1981). The common feature in all these instances is that the internalization of the ligand takes place through the coated pits. Coated pits were first observed by Roth and Porter (1964) in mosquito oocytes engaged in the uptake of extracellular yolk protein. Coated pits appear to continually bud into the cells to form coated vesicles. The major protein in coated vesicles is called clathrin ( $M_r$  180,000). Clathrin in association with two other proteins ( $M_r$  100,000 and 50,000) have the interesting property of organizing into basket-like structures (reviewed in Pearse, 1982). In some instances, such as that of LDL (Anderson *et al.*, 1977a) and asialoglycoproteins (Wall *et al.*, 1980) the receptors appear to be preclustered in the coated pits which are internalized regardless of ligand binding. In other instances, ligand binding apparently induces the lateral movement of the receptors into coated pits. Examples where such ligand-induced mobilization have been documented include the receptors for insulin, EGF and some viruses (reviewed in Pastan and Willingham, 1981; Helenius *et al.*, 1980).

The key role of the clustering of receptors into coated pits for efficient internalization was apparent from the study of a human mutation in the LDL receptor pathway where binding of LDL was normal but no internalization of the bound LDL occurred (Brown and Goldstein, 1976). Ultrastructural studies showed that the LDL receptors in the mutant cells were diffusely distributed on the plasma membrane and failed to be clustered in coated pits (Anderson *et al.*, 1977b; Carpentier *et al.*, 1979). On the basis of genetic studies of the family where this internalization-defective mutation occurred, Goldstein *et al.* (1977) concluded that the mutation was in the structural gene for the LDL receptor resulting in the formation of an altered receptor molecule where the domain responsible for interaction with a component of the coated pit was non-functional. Genetic studies of Miyaki *et al.* (1981) have essentially corroborated the findings of Goldstein *et al.* (1977). Therefore, a probable mechanism of clustering of receptors into coated pits of normal cells might include an interaction with a component of the coated pit. This interaction might help to anchor the receptor into the coated pits once it is carried there by normal lateral movement of the fluid membrane components perhaps aided by the participation of specific mechanoskeletal factors (Hemmaplardh *et al.*, 1974). Since receptors for many different ligands appear to cluster in the same coated pits, it is likely that they might share common domains for interaction with some element of the coated pits. In instances where apparent ligand-induced clustering of receptors into coated pits takes place it is not clear whether *de facto* cross-linking of receptors through multivalent ligands is the driving force for clustering or as in the case of LDL receptors the clustering is an innate property of the receptor itself. An alternative viewpoint suggests that ligand-binding induces an allosteric transition in the conformation of the receptors which leads to their recognition as a component of the coated pits (Pastan and Willingham, 1983). In this view, receptors which appear to cluster into coated pits regardless of ligand-binding are presumed to be in the conformation recognizable by some component of the coated pits. Further biochemical experimentation would be necessary to reconcile or distinguish between these divergent viewpoints.

Although there is general agreement as to the involvement of coated pits in receptor-mediated endocytosis, there is a controversy as to whether the coated pits physically detach themselves from the plasma membrane to form intracellular coated vesicles. The

traditional view, culled from electron microscopic observations in several (LDL, asialoglycoproteins, Semliki Forest virus), holds that free intracellular vesicles carry the ligand and the receptors into cells, the coated vesicles rapidly lose their clathrin coat to form smooth endocytic vesicles (called endosomes) and the clathrin coat is recycled back to the cell surface to form new coated pits (Goldstein *et al.*, 1979; Heuser *et al.*, 1980; Heuser and Evans, 1980). In contrast, Willingham and Pastan (1980) have shown that the apparent free coated vesicles do have thin convoluted connections to the plasma membrane. Therefore, they proposed that smooth endocytic vesicles (called receptosomes by them) are directly formed from coated pits and that the receptor does not get dissociated from the coated pits to be recycled.

### Recycling of cell surface receptors

Although the coated pit/endosome mediated internalization of ligands appears to be the general mechanism for receptor-mediated endocytosis, the intracellular destination of the ligand is by no means uniform. Some ligands such as LDL or asialoglycoproteins are delivered to lysosomes whereas others (maternal immunoglobulins, nerve growth factor) are destined for other compartments. The signals that mediate the direction of such intracellular vesicular traffic are not yet understood. Regardless of their final destination, at some point after internalization, the receptors and ligands must dissociate from each other. This dissociation seems to occur in endosomes which were shown to have an acidic pH (Tycko and Maxfield, 1982; Van Renswoude *et al.*, 1982). Maintenance of an acidic pH in endosomes appears to be mediated by an ATP-driven proton pump (Galloway *et al.*, 1983). Similar proton pumps have been found in isolated endosomal vesicles as well (Forgacs *et al.*, 1983; Stone *et al.*, 1983). Many ligands are known to dissociate from their receptors at pH below 6 (for instance, LDL, lysosomal enzymes, EGF, asialoglycoproteins and  $\alpha_2$ -macroglobulin). After dissociation from its ligand, the receptor is somehow transported to lysosome or other destinations where the ligand is transported. The receptor is often recycled back to plasma membrane where it might participate in multiple rounds of internalization.

Early kinetic studies on LDL uptake showed no apparent depletion of cell surface LDL receptors, while the cells internalized LDL at a continuous rate (Goldstein *et al.*, 1974, 1976). Therefore, at a steady state, the cell surface receptors that are internalized along with the ligand must be replaced at a constant rate. There are three possible sources for maintaining this constant number of cell surface receptors, *viz.* (i) synthesis of new receptors, (ii) receptors from a relatively large internal pool, and (iii) recycling of the receptors back to the cell surface following intracellular delivery of the ligand. Inhibition of protein synthesis does not affect the rate of internalization for at least 12 h (Brown and Goldstein, 1975; Brown *et al.*, 1982) and no large pool of LDL receptors have been found (Basu *et al.*, 1978; Schneider *et al.*, 1978) suggesting that LDL receptors are recycled back to the cell surface. In several other receptor systems incubation of cells with the ligand plus weak bases such as chloroquine or methylamine (which raise the lysosomal pH) led to a decline in the number of cell surface receptors. This disappearance was attributed to the trapping of receptors within lysosomes owing to the high pH of the organelle in the presence of the

weak bases (Tolleshaug and Berg, 1979; Gonzalez-Noriega *et al.*, 1980; Van Leuven *et al.*, 1980).

First direct evidence for receptor recycling was obtained in the LDL receptor system through the use of the carboxylic ionophores monensin or nigericin (Basu *et al.*, 1981). These ionophores disrupt proton gradients and raise the pH of the acidic intracellular compartments. Incubation of human fibroblasts with these ionophores in absence of the ligand led to a 50 % reduction in the cell surface LDL receptor activity suggesting that the receptors got trapped within the cell. The trapping of LDL receptors inside the cell was demonstrated by direct assay of LDL receptors in solubilized cells as well as by making the cells permeable to an antibody to the LDL receptor followed by indirect immunofluorescence. These studies also indicated that receptor entry occurs through the coated pits because monensin did not cause a depletion of cell surface LDL receptors from the internalization-defective cells whose receptors are not associated with coated pits. Furthermore, when fluorescent LDL was internalized in the presence of monensin, it accumulated in the same vesicles as the receptors which was visualized by staining with antireceptor antibody after permeabilizing the cells (Brown *et al.*, 1982). This suggested that monensin prevented the segregation of LDL and the receptor into separate compartments. Finally, receptor entry and recycling occurs continuously because monensin could trap 50 % of the receptors within 15 min inside the cell even when no LDL was present. Addition of LDL to such cells resulted in the disappearance of more than 90 % of the cell surface receptors. This curious effect raises the question whether in the absence of LDL only half of the cell surface LDL receptors enter the cell and recycle and the other half never enters the cell. Therefore, monensin could only trap half of the receptors in the absence of LDL. It is also possible that all the receptors enter and recycle and monensin slows the rate of return leading to a distortion in the steady-state so that at any point in time 50 % of the receptors remain in the cell. When LDL is added in the presence of monensin the ligand-bound receptors fail to be dissociated leading to a tighter intracellular trapping of the receptors. That the dissociation of ligand from the receptor is an important step in receptor recycling became apparent from the use of monoclonal and polyclonal antireceptor antibodies (Beisiegel *et al.*, 1981; Anderson *et al.*, 1982). While monoclonal antibodies did not affect the recycling process the polyclonal antibodies caused a rapid loss of cell surface LDL receptors. In contrast to the monensin effect this loss was irreversible. When the receptor-depleted cells were incubated in the absence of the polyclonal antireceptor antibody the cell surface receptors were replenished at the same rate as cellular protein synthesis and the replenishment was blocked by cycloheximide. Furthermore, monovalent Fab fragments of the polyclonal antibody were internalized but did not affect the recycling process. Similar results were obtained when a monoclonal antireceptor antibody was used. These findings suggest that both the polyclonal Fab fragments and the monoclonal antireceptor antibodies could be dissociated from the receptor in a prelysosomal compartment so that the receptors could be recycled whereas because of multivalent binding polyclonal antibodies do not dissociate from the receptor and the complex is delivered to the lysosomes where both the ligand and the receptor get degraded thereby causing irreversible loss of cell surface LDL receptors.

The pathway that a recycling receptor follows to get back to the cell surface is not yet clear. For mannose-terminal glycoproteins two intracellular pools of receptor-ligand

complexes was discerned from kinetic evidence—a base-sensitive (chloroquine/ammonium chloride) and a base-insensitive pool (Tietze *et al.*, 1982). Receptor complexes returned intact to the cell surface from the base-insensitive pool when complexes got dissociated in the base-sensitive pool from which the receptors return to the cell surface while the ligand was retained intracellularly. The partial recycling of LDL receptors by monensin alone is consistent with the notion of a dual pathway for receptor-recycling (Basu *et al.*, 1981; Brown *et al.*, 1982). The base-sensitive pool is likely to be in an intracellular acidic compartment such as endosomes. Similar dual pathways for the transit of receptor-ligand complexes resulting in distinct fates seem to exist for some asialoglycoproteins as well (Regan *et al.*, 1982; Schiff *et al.*, 1983). Double-label immunoelectron microscopic studies by Geuze *et al.* (1983) with antibodies against ligand, receptor and clathrin show that asialoglycoprotein receptors remained attached to the ligand in coated vesicles on the cell surface. Ligand and the receptor segregated in a set of tubular vesicles destined for the compartment of uncoupling of receptor and ligand (CURL). The receptors were found attached to the membrane of the CURL structures, whereas free ligand was found within the vesicle lumen. Moreover, the ends of the tubular extensions were coated with clathrin. These studies raise the possibility that the recycling receptors might cluster into these coated regions which then bud out excluding most of the contents to form small clathrin-coated vesicles. Such small coated vesicles with receptors embedded in their membranes may then find their way back to the cell surface. The ligands left in the lumen of CURL vesicles may be carried forward to lysosomes or other cellular structures. Ultrastructural and subcellular fractionation studies in a number of receptor systems (phosphomannosyl, LDL, EGF and insulin) have demonstrated that soon after internalization the ligands accumulate in derived vesicles (Khan *et al.*, 1982; Merion and Sly, 1983; Willingham and Pastan, 1983). From these vesicles the ligands were transferred to lysosomes in a time-dependent manner and this transfer could be blocked by ammonium chloride or monensin (Merion and Sly, 1983). It is interesting to note that the transfer of EGF from the derived vesicles to the lysosomes was much faster than that for LDL or phosphomannosyl ligand. Since EGF receptors are not reutilized this delay might represent the time needed for dissociation of the receptor-ligand complexes for the other two ligands.

Kaplan (1981) first pointed out that reutilization of receptors for multiple cycles of internalization of ligands appears to be a general rule for receptors whose function is ligand uptake to transport (i) extracellular nutrients (such as cholesterol in LDL or transferrin) or (ii) enzymes (lysosomal hydrolases) or (iii) unwanted proteolytic degradation (galactose, glucose or mannose-terminal glycoproteins,  $\alpha_2$ -macroglobulin-protease complexes). In contrast, receptors whose major function is information transfer through interaction with their ligands at the cell surface (such as receptor for polypeptide hormones insulin, EGF or nonhormonal humoral agents like immunoglobulin E) do not appear to be extensively reutilized. This distinctive behavior of the receptors depending on the nature of the ligand makes teleological sense, but continued uptake of hormones may not be necessary to generate the signal for hormone action. However, where the receptors serve a transport function for macromolecular carriers such as LDL, reutilization of receptors would increase the overall efficiency of the process ensuring a continuous intracellular delivery of the ligand.

## Conclusion

Biochemical, ultrastructural and genetic approaches in a number of systems have provided us with an unified picture of the process of entry of macromolecules into mammalian cells. We now know that coated pits serve as a mechanism for concentrating the randomly distributed cell surface receptors for efficient internalization of the ligand. An assortment of endocytic vesicles (coated and/or smooth) carry the receptor-ligand complexes into the cells. Rapid acidification of such intermediate vesicles result in the dissociation of the receptor-ligand complexes from which receptors could be recycled back to the cell surface for reutilization. Dissociated ligands in the lumen of the vesicle are often delivered to the lysosomes or other cellular compartments or even transported across the cell. Although the outline is clear several major controversies about the origin and direction of the vesicular traffic exist. The tremendous surge of activity in this field in the last few years permits the optimism that the dissection of the process of receptor-mediated endocytosis at the molecular level is not far off.

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## Expression of DNA transferred into mammalian cells

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**Abstract.** There are several methods to introduce purified DNA into mammalian cells. These include microinjection into the nuclei of the recipient cells and complexing the DNA with facilitating agents such as calcium-phosphate. After it enters the nucleus, the DNA is expressed in a large proportion of the cells. This expression is dependent upon *cis*-acting sequences that are recognized by the mammalian transcriptional and translational machinery. In a smaller proportion of cells, the exogenous DNA becomes covalently integrated into the host cell DNA at random sites. Non-selectable genes can be introduced into mammalian cells by ligating them to a selectable marker or mixing the DNA with carrier DNA containing a selectable marker. The DNA that is introduced into mammalian cells can be rescued for examination and analysis. These gene transfer methods have already proven to be useful in identification of sequences which are necessary for normal gene expression as well as gene regulation. In addition a number of genes have been isolated using gene transfer methods. DNA mediated gene transfer holds much promise to target genes to specific sites in the chromosomes, to understand mechanisms of mammalian development and cell differentiation and is expected to provide a method to produce important and novel gene products that may be used for diagnostic and therapeutic purposes.

**Keywords.** DNA transfer; gene expression; gene regulation; mammalian cell genetics.

### Introduction

A number of methods are currently available to introduce genetic information from one mammalian cell to another. Prominent among these methods is somatic cell hybridization (Kucherlapati and Ruddle, 1975). Intact cells from the same species or two different species can be readily fused with the aid of polyethylene glycol and proliferating hybrid cells can be generated. Such cell hybridization methods are extensively used to construct mammalian genetic maps, to study gene regulation at the cellular level and to produce monoclonal antibodies. Interspecies hybrids usually segregate the chromosomes of one of the parental types thus generating a series of hybrids each of which carries a subset of genetic information from the segregating parent. Though this feature is very useful and indeed forms the basis for gene mapping,

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Abbreviations used: TK, Thymidine kinase; UV, ultra-violet; HSV, Herpes simplex virus; LTR, long terminal repeat; DHFR, dihydrofolate reductase; MTX, methotrexate; CAT, chloremphenicol acetyl transferase; XPRT, Xanthine phosphoribosyl transferase; BPV, bovine papilloma viral; MMTV, mouse mammary tumor virus; MT, metallothionein; MEL, murine erythroleukemia; GaK, galactokinase.



it is not possible to control the level of chromosome segregation. Microcell chromosome mediated gene transfer permit introduction of partial genetic information from one cell into another but these methods also do not provide the opportunity to predetermine the amount and type of genetic information that can be introduced.

The advent of recombinant DNA technology now permits the isolation of virtually any gene sequence. Structural analysis of these DNA sequences can be readily performed and detailed restriction enzyme maps and nucleotide sequences (where necessary) can be obtained. A functional analysis of these DNA segments is facilitated by introducing them into mammalian cells. These DNA mediated gene transfer systems have become powerful tools to study mammalian gene expression and regulation in their normal host environment. In addition, these techniques are being used to initiate studies of mammalian development and differentiation. The methods also promise a rich future in which it may be possible to replace genes in mammalian cells and embryos with the attendant possibility of gene replacement therapy.

In this article we will describe the various methodologies of DNA mediated gene transfer, parameters that affect the introduction and expression of these genes, use of these methods to isolate DNA sequences and finally some perspectives on the future of these methodologies. The references we provide are not intended to be exhaustive but have been chosen to illustrate specific aspects of the systems. In addition, there is a large body of literature on the use of the DNA transfer systems to study aspects of malignancy in mammalian cells which is not included in this article. For recent reviews on this subject please see (Varmus 1982, Cooper, 1982).

### Methods of gene transfer

The feasibility of introducing DNA into bacterial cells by a variety of natural and artificial methods has enhanced the ability to conduct genetic analysis of several prokaryotic organisms. A similar development of gene transfer techniques in mammalian cells has been achieved over the past few years. As in the case of bacterial systems, the methods of gene transfer can be classified into natural and artificial systems. The most common natural method of introduction of foreign DNA is achieved by infection of cells with intact viruses. If the cell type is permissive the viral DNA undergoes replication and new virus particles are synthesized causing eventual cell death. The viral DNA gets integrated into the host cell DNA and expressed as part of the host genome in case of nonpermissive cell type. The viral genomes utilize the host cell machinery for transcription and translation and cause a variety of changes in the host cells through mechanisms which are not completely understood. The viral genomes are now being used as vectors to introduce purified genes into mammalian cells. This aspect would be discussed later.

One set of these viral transfection methods had an important impact on future developments in gene transfer experiments. Munyon *et al.* (1971) have infected normal L-cells deficient in the pyrimidine salvage pathway enzyme thymidine kinase (TK) with ultra-violet (UV) inactivated Herpes simplex virus (HSV) type 1. This DNA contains a gene for thymidine kinase. They were able to show that the cells can acquire and express the viral TK gene. Similar experiments were conducted by Davidson

(1973). These observations paved the way for future use of the TK gene transfer system in DNA transfection studies.

The artificial methods of gene transfer can be classified into direct and indirect methods. The direct methods involve injection of the appropriate DNA into the nucleus of the recipient cell and the indirect methods usually involve the complexing of DNA with facilitating agents and presenting this complex to cells.

#### *Direct methods*

Based upon a microinjection procedure originally developed by Diacumakos *et al.* (1970), Anderson *et al.* (1980) have microinjected purified DNA into the nuclei of mouse L-cells. In these experiments, Anderson and colleagues used a cloned fragment of HSV DNA containing the TK gene. TK<sup>+</sup> cells were isolated by the use of the HAT selection system (Szybalska and Szybalski, 1962). The microinjection procedure was found to be quite efficient in that one cell in twenty was stably transformed. The fact that these cells could grow stably in the selective medium indicated that they have acquired and are expressing the exogenously introduced gene. Capecchi (1980) has carefully examined the factors that influence the stable acquisition of DNA by microinjection procedures. He observed that transformation efficiency was relatively insensitive to DNA concentration and did not depend on the presence of non specific carrier DNA. He has also noted that the presence of SV40 DNA replication origin sequences enhanced the stable transformation efficiencies. It is now known that this is most probably due to the presence of transcriptional enhancer signals associated with the early promoter of SV40 which is located in the 'ori' region of this viral genome.

The most extensive and widespread use of microinjection is currently in introducing purified DNA sequences into mammalian embryos. Gordon and Ruddle (1981) have developed a method to microinject DNA into the pronucleus of a fertilized mouse oocyte. The injected embryos are transferred to the uterus of a pseudopregnant female. In a number of cases the implanted embryos develop into complete mice, several of which were found to contain the injected DNA sequences. This method is gaining widespread use and promises to provide a method to follow the expression of a defined gene through all stages of development and differentiation. Results obtained from these experiments will be discussed later.

#### *Indirect methods*

Among the indirect methods of gene transfer the most commonly used system is that of coprecipitation of DNA with calcium phosphate. Though a method of introducing naked DNA into cultured mammalian cells was described by Szybalska and Szybalski (1962), the widespread use of DNA transfection techniques had to await future developments. In 1973 Graham and Vander Eb developed a method to increase the infectivity of Adenovirus DNA by complexing it with calcium phosphate. In this method, the DNA is coprecipitated with calcium phosphate and the resulting suspension is then added to cells. The cells were allowed to incubate for variable periods of time following which the precipitate is removed and the cells subjected to growth and analysis. The widespread use of this method of DNA transfer followed the demonstration that purified DNA from HSV can confer a TK<sup>+</sup> phenotype to LMTK<sup>-</sup> cells

(Wigler *et al.*, 1977; Maitland and McDougall, 1977). These two groups have shown that HSV DNA digested with certain restriction endonucleases loses its ability to confer a TK<sup>+</sup> phenotype while other enzymes failed to do so. Knowledge of enzymes which failed to destroy the TK activity in HSV1 DNA permitted isolation of a 3.4 Bam HI fragment which alone was capable of conferring the TK<sup>+</sup> phenotype to TI cells. This was the first gene that was isolated by the use of DNA transfection techniques. Different groups of workers have since used this method to successfully introduce different genes into different recipient cell lines.

DNA complexed with polycations such as poly L-ornithine (Farber *et al.*, 1975), DEAE Dextran (Pagano, 1970; Farber *et al.*, 1975) is also used to transfect cells. These methods are generally used to transfect viral DNA sequences and have proven to be useful in increasing both uptake and expression of foreign DNA sequences. Milne and Herzberg (1981) have, however, reported that L-cells transfected in this fashion show a high level of transient expression but failed to yield stable transformants. This aspect needs further investigation.

Schaefer-Ridder *et al.* (1982) have used liposomes as gene carriers. Stable transfection of mouse L-cells deficient in thymidine kinase was achieved by liposome mediated transfer of a recombinant plasmid carrying the thymidine kinase gene. 10 per cent of the recipient cells expressed thymidine kinase activity. Human HGPRT gene was transferred into HGPRT deficient mouse cells with a frequency of approximately  $1 \times 10^{-5}$  using lipochromosomes (Mukherjee *et al.*, 1978; Hoffman *et al.*, 1981).

Schaffner (1980) has demonstrated that cloned genes can be transferred from bacteria to monkey cells directly by fusion of lysozyme-treated bacteria (protoplasts) with monkey cells in the presence of polyethylene glycol. Later other groups of workers (Sandri-Goldin *et al.*, 1981; DeSaint Vincent *et al.*, 1981) used this method to successfully introduce foreign genes into mammalian cells. In optimal conditions, transfer by fusion seems to be 10 to 20 fold more efficient than DNA transfection. Ca-PO<sub>4</sub> coprecipitation technique (Rassoulzadegan *et al.*, 1982).

It is known that not all mammalian cells respond equally well to each of the methods of gene transfer. It has been found that some cells are quite resistant to Ca-PO<sub>4</sub> mediated gene transfer while they are more easily transferable by protoplast fusion. A possible disadvantage with the protoplast fusion is that in addition to the desired gene the bacterial DNA will also be introduced into the mammalian cell.

A number of alternative methods to circumvent the problems with each of the above methods are being examined. One method that is of promise is electroporation. Zimmerman and Vienken (1982) and Neuman *et al.* (1982) have shown that cell fusion can be achieved quite efficiently if a high voltage of electricity is discharged in solution containing the appropriate mixture of cells. This method is modified with naked DNA is mixed with the desired recipient cells and pulsed with high voltage electricity. This method is now said to be quite useful in transfecting cells which are known to be resistant to transfection by other methods (Potter, H. and Leder, personal communication).

Choice of the methods of gene transfer depends on the nature of DNA to be introduced. Use of these methods requires a knowledge of the nature of cell types, size and physical state of the DNA to be transferred and the experimental conditions. Several factors play a role in the efficiency with which a gene can be transferred.

### Factors that affect efficiency of gene transfer

Different methods of gene transfer are suited for different purposes. Each of the methods has limitations and advantages. Knowledge about these features would greatly facilitate the appropriate choice of cell type and gene transfer method.

In cases where whole viruses are used, the recipient cell type plays a very important role. Not all cells are susceptible to the viral infection. For example, the DNA tumor virus SV40 can infect a number of mammalian cells (e.g., monkey, human and mouse) but other viruses have a more limited host range. It is also important to note that the fate of the viral nucleic acid, once it enters the cell is also dependent on the host. SV40 can replicate in monkey cells resulting in eventual cell death while mouse and human cells are nonpermissive for replication of the viral DNA.

The microinjection method is well suited for embryos and several cell types that grow attached to a substratum where the target cell nucleus is easily visible. Cells that grow in suspension are not extremely suitable for microinjection. In general, if the number of target cells is limiting, microinjection may prove most appropriate. In this direct method, physical constraints such as size of the cell and size of the nucleus play an important role in the ease with which DNA can be introduced.

Methods that involve the preparation of protoplasts yield a very high number of transfectants but such a method obviously involves prior preparation. Similarly, liposome mediated transfer also involves manufacture of liposomes and incorporation of the DNA before they can be used.

The calcium-phosphate co-precipitation of DNA and the DEAE-dextran packaging of DNA are among the most widely used methods of DNA transfer. This is probably due to the ease with which these facilitating agents can be used. A variety of factors seem to influence the efficiency with which cells can be transfected with the aid of these agents.

One of the most important factors that affect the efficiency of transfer of genes into new cellular environments is the nature of the recipient cell line. Colbere-Garapin *et al.* (1981) demonstrated that following transfection with kanamycin resistance gene linked to the HSV1 TK promoter region, several mammalian cell lines (murine, simian and human) become resistant to G-418, an analogue of kanamycin. But the efficiency of transfer of this drug resistance was different in different cell lines. Graf *et al.* (1979) have also shown that transformation frequency is critically dependent on the particular line used as recipient. Mouse L-cells have been widely used as recipients for gene transfer. Although only limited information is available, it appears that L-cells act as good recipients while in cells like chinese hamster ovary cells, the frequencies of DNA transfer are 1/10 those for L-cells (Srinivasan and Lewis, 1980).

Cells of the same species and even cells from a single clone show differences in the efficiency of gene transfer (Corsaro and Pearson 1981a,b). The transformation efficiency for TK was found to vary 10-20-fold among different subclones of the LM TK-mouse fibroblast cell line.

Specific features of recipient cell membrane also play an important role in the transformation frequency. Lewis *et al.* (1980) and Lowy *et al.* (1978) have shown that post transfection shock of cells with DMSO enhanced the efficiency of DNA transfer by calcium-phosphate precipitation method. The optimum concentrations of DMSO

and time of exposure are different for different cell types and has to be determined for each cell line. Such DMSO shocks are now routinely used by different laboratories.

Graham and Vander Eb (1973) found that the efficiency of transfection by calcium chloride technique could be enhanced by the use of carrier DNA. Our own studies (Noonan, K. and Kucherlapati, R. S., unpublished results), indicated that transfection of HSV TK gene can be enhanced 10-fold by using salmon sperm DNA as carrier. It has been shown by Perucho *et al.* (1980b) also that transformation is much less efficient when carrier DNA is omitted. It is clear, however, that there is no absolute need for carrier DNA (Linnenbach *et al.*, 1980; Huttner *et al.*, 1981). It has been shown that selectable genes introduced with a carrier DNA form a high molecular weight molecule termed a transgenome (Ruddle, 1979, 1980; Scangos *et al.*, 1981) or Pekalosome (Perucho *et al.*, 1980b). The selectable gene becomes associated with the carrier DNA to form the transgenome and this association has been directly demonstrated by Perucho *et al.* (1980b). Transgenomes are initially unstable and are lost from the population. After variable generations these transgenomes become stable and are found to be associated with the recipient cell chromosome (Scangos *et al.*, 1981).

Several experimental results address the questions about the possible roles of carrier DNA in transfection. One of the possible roles of carrier DNA is that it provides functional replication origin to the selectable DNA permitting replication of the complex until it is integrated into the host genome. This view is supported by the observation that eukaryotic DNA acts as a better carrier than prokaryotic DNA. Studies carried out by Capecchi (1980) which showed that SV40 containing 'ori' region increased the efficiency of transformation 100-fold supports this view. It is also possible that carrier DNA contains some enhancer sequences like several viruses (Gruss *et al.*, 1981; Benoist and Chambon, 1981) and these sequences enhance transfection efficiencies when selectable genes become ligated to carrier DNA in the proximity of such sequences.

Another possible role of carrier DNA is that it is providing sites of integration into the host genome by homologous recombination. It is possible that integration of transgenome occurs as a result of recombination between unique or reiterated sequences present in carrier DNA and homologous sequences present in the recipient cell genome. This view can be supported by some of our experimental results which shows that carrier effects can be mimicked if plasmid carrying a chinese hamster Alu family of repeated sequence is used without any additional carrier DNA (Kucherlapati, R., Jelinek, W., Krauter, K. and Leinwano, L., unpublished results). Further experiments are necessary to prove that association of transgenome with the recipient cell genome is the result of homologous recombination events.

Besides all these factors so far discussed, another factor affecting the efficiency of gene transfer is the nature of the DNA introduced. It has been found that linearized plasmid DNA is five times more efficient in transferring the TK<sup>+</sup> phenotype to mouse cells than its circular counterpart (Colbere-Garapin *et al.*, 1979). It is now known that mammalian somatic cells are quite efficient at end-to-end joining of DNA segments and that linear DNA molecules are more recombinogenic (Wilson *et al.*, 1982; Subramanian, 1979). This knowledge may provide the basis for higher transformation frequency with linearized plasmid.

Thus efficiency of gene transfer depends on a number of factors and elucidation of

the different factors will provide the opportunity to devise methods for high frequency of transfer of any gene into any mammalian cell type.

### Fate of the DNA in the recipient cells

We have already documented the fact that mammalian cells are able to take up exogenously added DNA and express genes included on that DNA. Different DNA-mediated gene transfer methods proved to be useful in introducing foreign DNA into mammalian cells. Several investigators have made attempts to study the fate of the exogenous DNA in the recipient cell.

The most commonly used method of gene transfer is co-precipitation of DNA with calcium phosphate. DNA forms a tight complex with calcium phosphate and becomes resistant to nucleases present in serum or added externally. Under optimal conditions, all of the recipient cells take up CaPi-DNA complex but ultimately only a fraction of the cells have detectable CaPi-DNA complex in the nucleus (Loyter *et al.*, 1982). The exact mechanism by which DNA makes its way into the nucleus is not clear. It can be suggested from the results that the movement of the DNA from the cytoplasm to the nucleus constitutes the most significant barrier to gene transfer. To increase the efficiency of gene transfer it is necessary to facilitate entry of DNA from cytoplasm to the nucleus and no expression is possible if it is injected into the cytoplasm by microinjection procedure (Capecchi, 1980). After entering the nucleus several possible fates may await the foreign DNA. The transforming element may persist within the nucleus as an autonomously replicating extrachromosomal unit or it is stably integrated into a host chromosome.

The possibility that the transforming DNA may exist as an autonomously replicating extrachromosomal element depends on the nature of the vector used. For example, vectors containing SV40 replication origin and an intact *A* gene can replicate autonomously in monkey cells (Hamer *et al.*, 1979). A number of genes have been cloned *in vitro* with SV40 vectors and propagated successfully as autonomously replicating molecules in cultured monkey kidney cells. Similarly vectors containing polyoma sequences replicate autonomously in mouse cells (Colbere-Garapin *et al.*, 1981). Foreign DNA linked to bovine papilloma viral DNA also persists as an extrachromosomal element in several mammalian cells (Dimaio *et al.*, 1982). If the DNA is unintegrated it may not segregate equally into the progeny cells after each cell division and may result in the loss of the phenotype among a certain percentage of cells in the population. It is also possible that integrated DNA is excised during early stages of the development of the cell line causing instability in the phenotypic expression of the transferred gene. These views are supported by results reported by Pellicer *et al.*, (1980); Ostrander *et al.* (1982); Davies *et al.* (1982). The autonomous replication pattern of any DNA in recipient cells can be deduced by isolating low molecular weight DNA from the cells.

Different laboratories studied the fate of exogenous sequences in the recipient cells and found that foreign DNA is stably integrated into the host DNA. The integrated DNA can be isolated as high molecular weight form of DNA and can be detected by appropriate restriction enzyme digestion and hybridization using the foreign DNA as

probe. Pellicer *et al.* (1978) examined the fate of the TK gene in several independent clones after transfecting mutant mouse cells (LTK<sup>-</sup>) deficient in thymidine kinase with BamHI restriction endonuclease cleaved HSV1 DNA. Examination of the clones showed the TK gene is present in all cells at a frequency of one copy per chromosomal complement and is stably integrated in the DNA of all the transformants. The integration is not site specific and occurs at different regions of the recipient cell DNA.

When a selectable gene is transferred with carrier DNA, cells which incorporate selectable markers are also likely to incorporate sequences from the carrier DNA. As discussed before, the selectable gene becomes associated with carrier DNA to form a high molecular weight molecule termed a transgenome. This transgenome is maintained stably and after a few generations is found to be integrated into the recipient cell chromosomes (Scangos *et al.*, 1981). This has been demonstrated directly by Perucho *et al.* (1980b) using salmon DNA as carrier DNA for the HSV TK gene. They showed that host cell ligates incorporated DNA into a large concatameric structure which is finally integrated in a stable fashion within the high molecular weight nuclear DNA of the host cell. The studies by different groups of workers (Huttner *et al.*, 1979; Wigler *et al.*, 1980) indicated that virtually any purified sequence can be introduced into mammalian cells by cotransformation with a selectable gene. The exogenous DNA integrates into the host genome and the selection of the selectable gene is necessary for the maintenance of nonselectable sequences. Pellicer *et al.* (1980) have shown that in buffalo rat liver cells cotransformed with a growth hormone gene (hGH), the foreign gene is inserted into particular chromosomes and the site of insertion is invariant within a given cell line. Different lines, however, contain the transforming element on different chromosomes. The site of insertion is not restricted to a unique chromosome. It is apparent from these experiments that TK or hGH sequences are not directing the site of insertion. In another set of experiments (Robins *et al.*, 1981) a series of rat liver cell lines cotransformed with a variant human growth hormone gene were examined by *in situ* hybridization; it was found that in most of the cell lines the cotransformed sequences reside in a chromosome of the host cell and each line revealed a different site of integration for the transforming sequences. After integration into the host genome the foreign DNA may undergo some rearrangements. We (Hwang and Kucherlapati, 1983) studied the organization of integrated SV40 sequences in an uncloned population of a transformed human fibroblast cell line and found that following the initial integration event, viral as well as the flanking host DNA sequences become unstable and are subject to deletions and rearrangements. This instability is for a short period after which the integration of SV40 is stable and maintained for a number of generations.

The fact that the foreign DNA sequences seem to integrate at random sites has raised the issue of the ability of mammalian cells to mediate homologous recombination. Several earlier experiments to detect homologous recombination have failed to yield positive results (Rosenstrauss and Chasin, 1978; Tarrant and Holliday, 1977). This question has been reinvestigated by the gene transfer methods. The general strategy of many of these experiments is to introduce two mutant, non complementing, selectable genes into mammalian cells and select for events which result in reconstruction of an intact gene by homologous recombination. DeSaint Vincent and Wahl (1983) analyzed the fate of two recombinant plasmids containing overlapping fragments of a cloned Syrian hamster CAD gene introduced into chinese hamster cells. They found that the

mammalian cells catalyzed homologous recombination between the two plasmids resulting in the reconstruction of an intact gene. The recombined gene sequences however integrated into the host chromosome at random sites. Results obtained by Folger *et al.* (1982), Small and Scangos (1983), Shapira *et al.* (1983), Miller and Temin (1983) have also shown that mammalian cells have the ability to mediate homologous recombination between exogenously introduced plasmids. We have recently shown that homologous recombination between plasmid molecules can be enhanced in mammalian cells if one of the plasmids is linearized by introducing a double-stranded cut within the region of homology (Kucherlapati *et al.*, 1984).

In addition to the homologous recombination events, foreign DNA is also subject to non homologous recombination events. Subramanian (1979) has shown that linear molecules tend to join end-to-end irrespective of the nature of the ends. More extensive results of this type have been provided by Wilson and colleagues (Wake and Wilson, 1979, 1980; Wilson *et al.*, 1982).

All of these results indicate that perhaps the DNA introduced into mammalian cells is highly recombinogenic and can undergo homologous or non homologous recombination events forming concatemeric molecules or transgenomes. This DNA then integrates into the host cell chromosome by mechanisms which are currently not well understood.

### Vectors for introduction of DNA

The development of DNA-mediated gene transfer systems permits the introduction of a number of DNA sequences into mammalian cells. As a consequence, a variety of DNA vectors have been developed which will propagate and express covalently linked genes in different recipient cell types.

The transfer of foreign genes need not depend upon the expression of the gene of interest in the recipient cells. Rather, one may ensure its presence by introducing such a nonselectable DNA sequence together with a selectable marker into recipient cells. A frequently used selectable marker is the herpes virus TK gene. This system has been chosen because  $TK^+$  phenotype can be efficiently selected over a  $TK^-$  background by utilizing growth conditions in which the pyrimidine salvage pathway enzyme, thymidine kinase, is necessary for survival. There exist cell lines deficient in TK with low rates of spontaneous reversion to the  $TK^+$  phenotype which can be used as recipients. The viral protein is well characterized and is readily distinguishable from the cellular enzyme. Maitland and McDougall (1977) and Wigler *et al.* (1977) have shown that purified DNA from HSV types I or II can confer the  $TK^+$  phenotype to  $TK^-$  cells when the DNA was presented to the cells as calcium phosphate coprecipitate. Several groups of investigators (Wigler *et al.*, 1977; Minson *et al.*, 1978) have shown that the functional HSV TK gene is located within a 3.4 kb DNA fragment that can be isolated from the HSV genome by Bam HI restriction enzyme digestion and can be used to stably transfect  $TK^-$  cells to the  $TK^+$  genotype. The 3.4 kb Bam HI fragment has been cloned into the prokaryotic plasmid pBR322 and the cloned TK gene retains the capacity to transfect  $TK^-$  cells (Colbere-Garapin *et al.*, 1979; Enquist *et al.*, 1980; McKnight *et al.*, 1979; McKnight, 1980).

Colbere-Garapin *et al.* (1981) have used TK gene to construct a vector in which the



promoter region of the HSV type I TK gene has been linked to the gene coding for the aminoglycoside 3'-phosphotransferase coded for by the Tn5 transposon. This enzyme makes the cells resistant to the antibiotic G-418, which is otherwise toxic to the cells. They found that several mammalian cell lines (murine, simian and human) became resistant to G-418 after transfection with this recombinant plasmid and in this case it can be shown that transcription is initiated using the TK promoter.

Palmiter *et al.* (1982) used the structural gene of herpes virus thymidine kinase in constructing a fusion plasmid pMK by fusion with mouse metallothionein 1-promoter regulator region. pMK was introduced into mouse embryos by microinjection followed by reinsertion of the eggs into foster mothers. pMK sequences were detected in 15 % of the mice and seven of them showed high level of viral thymidine kinase in liver. In this case, TK gene was expressed using the mouse metallothionein promoter.

We have constructed a chimeric plasmid containing a DNA fragment from the genome of Moloney murine leukemia virus including the viral LTR and covalently linked HSVI-TK gene whose promoter was removed. This hybrid DNA structure was introduced into TK<sup>-</sup> mouse cells and was found to express TK<sup>+</sup> phenotype. We were able to show that transcription of the gene is initiated in MOMLV LTR (Gilboa *et al.*, 1982).

In the cases described above, the ability to introduce the foreign DNA is dependent upon the lack or mutant activity of a native gene. This problem has been circumvented by the use of gene sequences which are easy to assay or which provide a dominant selectable system.

Dihydrofolate reductase (DHFR) gene is such a dominant selectable marker and presence of this gene in a mutant form or high copy number confers resistance to high levels of the folate antagonist methotrexate (MTX) (Christman *et al.*, 1982; Schimke *et al.*, 1978; Wigler *et al.*, 1980). Murray *et al.* (1983) constructed a biologically active DHFR chimera by placing transcriptional promoter of the Harvey sarcoma virus long terminal repeat at the 5'-end of a DHFR cDNA. This chimera was dominant acting and was able to confer a methotrexate-resistant phenotype on wild type NIH 3T3 cells. The use of this gene or other more convenient drug resistance markers such as the bacterial gpt gene (Mulligan and Berg, 1980) may permit transfer and expression of virtually any genetic element into a variety of new cellular environments.

The use of bacterial genes which do not have a eukaryotic counterpart has become an important alternative method to transfer genes into mammalian cells. The bacterial genes that are commonly used for this purpose are chloremphenicol acetyl transferase (CAT), Xanthine phosphoribosyl transferase (XPRT or gpt) and the bacterial neo gene. The CAT gene product can convert chloremphenicol to its acetylated products each of which can be readily distinguished by thin layer chromatography. Gorman *et al.* (1982) have constructed plasmids in which the CAT gene was placed under the transcriptional control of SV40 or RSV promoters. The CAT gene, though not selectable, can be easily assayed and has become an important tool in functional assays for promoters and other *cis* acting regulatory sequences.

Mulligan and Berg (1980) constructed chimeric plasmids in which the bacterial gpt gene was placed under the control of the transcriptional signals of SV40. This plasmid can be introduced into mammalian cells and its expression can be selected for by the use of mycophenolic acid and xanthine. When this plasmid is introduced into HPRT<sup>-</sup> cells,

medium containing HAT can be used to select for the gpt gene expression. The basic vector into which the gpt gene was introduced was used by Southern and Berg (1982) to obtain bacterial *neo* gene expression plasmid. This gene when expressed in mammalian cells confers the cells resistance to the aminoglycoside G418. All of these SV40 vectors are quite extensively used for gene transfer purposes.

Different DNA and RNA tumor viruses are good candidates for use as vectors for the introduction of foreign DNA into animal cells because many of these viruses have been studied extensively and they could either replicate to give progeny or integrate their DNA into host chromosome depending on recipient cells. Since the viral genome includes strong promoters, it is possible to ensure efficient expression of the foreign DNA carried on it and hence efficient production of foreign gene products.

Two approaches have been exploited to utilize viral nucleic acid sequences as vectors. The first is to make recombinants between viral DNA and a piece of foreign DNA and then to pass the hybrid molecule through permissive cells in the presence of helper virus to produce encapsidated virions containing the recombinant DNA. Alternatively, recombinants between virus and foreign DNA can be constructed and used directly for transfection. A number of different gene transfer vectors are now available. Each of these vectors have different properties which make them useful in different cell types. The nature of some of the vectors and their specialized properties are described below.

#### *Autonomous replication vectors*

SV40 is one of the most commonly used viral vector for introducing foreign DNA sequences into mammalian cells. SV40 is particularly attractive as a transducing vector for various reasons. The viral genome consists of a single, small, covalently closed circular DNA molecule whose entire nucleotide sequence has been determined (Reddy *et al.*, 1978; Fiers, 1978); the genomic regions responsible for the various viral functions have been accurately located with respect to the detailed physical map of the DNA (Reddy *et al.*, 1978). The genome can be divided into early and late regions; the early region is expressed throughout the lytic cycle and encodes the *T* antigens which are responsible for malignant transformation of nonpermissive cells as well as for initiation of viral DNA replication in permissive cells. The late region encodes the viral structural proteins and in between the early and late regions there is a DNA sequence containing the origin of viral DNA replication. In COS cells (Gluzman, 1982) any piece of DNA containing a SV40 origin of replication will replicate autonomously because the cells produce SV40 *T* antigen in a constitutive fashion. A number of genes have been cloned *in vitro* with SV40 vectors and propagated successfully as extra-chromosomal elements in cultured monkey kidney cells. For example Hamer *et al.* (1979) inserted rabbit  $\beta$ -globin complementary DNA (cDNA) into SV40 DNA in place of the gene coding for the virus major capsid protein VPI. The recombinant genome multiplied efficiently in CV1 monkey kidney cell cultures and was transcribed to yield cytoplasmic, polyadenylated hybrid mRNAs containing the  $\beta$ -globin coding sequence. Cells propagating the recombinant plasmid produced substantial quantities of rabbit  $\beta$ -globin polypeptide. Other genes which have been cloned *in vitro* with SV40 vectors and introduced in monkey cells include segments of phage DNA (Ganem *et al.*, 1976). *Escherichia coli* DNA coding for tRNA Tyr (Hamer, 1977), and guanine phosphoribosyl transferase (Mulligan and Berg, 1981).

Bovine papilloma viral (BPV) DNA has been found to replicate autonomously in susceptible mouse cells (Law *et al.*, 1981); no integration of BPV-1 genome in the host chromosome was detected when mouse C127 cells were transformed with BPV type I virions. Sarver *et al.* (1981) demonstrated that a novel eukaryotic vector derived from the transforming region of BPV is highly effective for introducing foreign genes into mammalian cells. They constructed a DNA hybrid molecule BPV<sub>69T</sub>-rI containing the transforming region of BPV DNA and the rat preproinsulin gene I (rI1) and used it to transform mouse cells. DNA hybridization analysis revealed the presence of multiple unintegrated copies of hybrid DNA molecules with the BPV1 DNA segment and the rI1 gene covalently linked in selected transformed cell lines. BPV DNA has been suggested as potential vector because BPV transformed cells contain multiple copies (10 to 20 per cell) of the viral DNA which exist as plasmid molecules and they are efficient in inducing transformed foci in susceptible mouse cells (Howley *et al.*, 1980; Lowy *et al.*, 1980a). Since BPV DNA does not integrate into host genome, the physical contiguity of the foreign DNA segment should be preserved.

DiMaio *et al.* (1982) have shown that BPV vectors can be propagated as a plasmid in both bacterial cells and mouse cells. They constructed a BPV-derived recombinant plasmid composed of a subgenomic transforming fragment of BPV DNA, a deletion derivative of pBR322 and a 7.6 kilobase fragment of DNA from the human  $\beta$ -globin gene and found that this plasmid propagated as an extrachromosomal element in both mouse and bacterial cells with a copy number of about 10–30 per cell. The ability of BPV plasmids to act as shuttle vectors between bacterial and mammalian cells provides a rapid means of recovering and analyzing foreign genes introduced into mammalian cells.

Polyoma virus replicates autonomously in mouse cells but integrates into the host cell DNA if the host is a nonmouse cell. O'Hare (1981) has utilized a polyoma vector successfully for the introduction and expression of the bacterial neomycin gene in mouse cells. This vector can be used to transfer any foreign gene as extrachromosomal element into mouse cells.

#### *Double or multiple vectors*

Some vectors can be constructed in such a way that they would permit expression of two or more genes. SV40 and retroviral vectors can be used for this purpose. SV40 has two sets of genes and it is possible to delete one and to replace it with foreign DNA and introduce it into the mammalian cells along with helper virus to provide essential viral functions. The viral DNA would provide the promoter, poly A addition signal and the splice signals when necessary.

Retrovirus vectors are now drawing more attention because they possess several advantages. They can be readily introduced into cultured cells as well as laboratory animals and it efficiently integrates into the chromosomes of the host cell. Up to 7.5 kb of nonretroviral coding information can be packaged and so more than one gene can be packaged into the vector. Retroviruses have a genome from which a single primary transcript is made which is processed in two alternative forms yielding two separate mRNAs each coding for a different protein. This characteristic of the virus can be utilized in using it as a double vector. The viral gene can be replaced with any foreign

DNA sequence for expression in mammalian cells. It has been shown by Joyner *et al.* (1982) that long terminal repeats (LTRs) of a murine retrovirus can activate expression of heterologous gene coding sequence from which a functional promoter region has been deleted. Gilboa *et al.* (1982) constructed hybrid DNA containing 0.9 kb DNA fragment from the genome of Moloney murine leukemia virus, including LTR and HSV I TK gene whose promoter was previously removed. The hybrid DNA was expressed when introduced into the chromosome of TK<sup>-</sup> mouse cells and expression of the TK gene was found to be dependent on functions provided in *cis* by the viral DNA fragment. In a second series of experiments Gilboa (1983) replaced the neo viral *gag* and *env* genes with HSV TK and bacterial neo genes and both of the genes were expressed.

### Regulatable vectors

Different viral sequences may respond to different regulatory signals and this feature can be utilized to construct vectors containing the sequences which can be regulated and hence the expression of the gene cloned into the vector can be regulated after introduction into the recipient cells.

Huang *et al.* (1981) have used a chimeric plasmid in which the foreign gene is ligated to a mouse mammary tumor virus promoter. They have shown that the level of the foreign gene expression can be regulated by glucocorticoids and the sequences necessary for hormonal control are specified by the viral genome carrying the foreign gene and probably map within the viral LTR. They constructed chimeric molecules between p21 transforming gene of Harvey murine sarcoma virus and DNA containing the long terminal repeat of mouse mammary tumor virus (MMTV) and transfected NIH3T3 cells with the hybrid DNA. The levels of p21 RNA and protein in the transformants were found to be regulated by physiological concentrations of dexamethasone, a synthetic glucocorticoid. In addition, the transcripts were shown to originate at the normal MMTV transcription initiation site. Lee *et al.* (1981) have provided evidence that MMTV LTR contains sequences which are sufficient for glucocorticoid regulation of gene expression. As additional knowledge about these regulatory sequences become available, it will be possible to construct vectors with more defined functions.

Another sequence which provides for regulation of gene expression is the mouse metallothionein (MT)-I promoter. The MT gene expression can be induced by heavy metals. It has been shown that expression of HSV thymidine kinase gene joined to mouse MT-1-promoter is regulated by cadmium when it is either transfected into mouse L-cells (Mayo *et al.*, 1982) or injected into mouse eggs (Brinster *et al.*, 1982).

Chao *et al.* (1983) have shown that some specific DNA sequences within or flanking the  $\beta$ -globin genes are involved in the activation of globin genes in murine erythroleukemia (MEL) cells by chemical inducers such as dimethyl sulphoxide. MEL cells were transfected with hybrid mouse-human  $\beta$ -globin gene as well as intact human  $\beta$ -globin gene and those genes were regulated appropriately during differentiation of MEL cells in culture. The addition of chemical inducers to the co-transformed cells resulted in a 5 to 50 fold increase in the level of mRNA transcribed from the exogenous globin gene and experiments indicated that induction of hybrid mRNA resulted at least

in part from the increase in the rate of globin gene transcription and the induction appeared to be specific for globin genes within an erythroid cell.

Thus, understanding of the DNA sequences involved in gene regulation can be utilized in the construction of the vectors containing those sequences for efficiently introducing and regulating genes in different mammalian cell types.

### *Amplifiable Vectors*

There are several genes which are amplified in mammalian cells and one of these well characterized genes is the DHFR gene. This gene is dominant and amplifiable in wild type mouse cells.

Murray *et al.* (1983) constructed a recombinant DNA plasmid in which the transcriptional promoter of the Harvey sarcoma virus long terminal repeat was at the 5'-end of a mouse DHFR cDNA. This chimeric sequence mediated MTX resistance when introduced into mouse 3T3 cells and was amplified when the MTX resistant transfectants were selected to grow in increasing concentrations of MTX. Mouse DHFR gene linked to MMTV promoter is also expressed and amplified in response to the addition of MTX (Lee *et al.*, 1981). Another well characterized amplifiable gene is the multifunctional hamster gene referred to as the CAD gene which shows amplification in response to the drug PALA.

All these different types of vectors so far discussed proved to be efficient in transferring genes into the mammalian cells. Understanding of the role of different DNA sequences in the expression of the gene will provide additional opportunities to construct vectors for introduction and efficient expression of any foreign gene in any mammalian cell type.

### **Expression of transfected genes**

The expression of DNA sequences introduced into mammalian cells can be inferred by indirect methods such as the selective advantage they may confer on the cells or more directly at the level of RNA and or protein. Such expression assays permit identification of *cis* and *trans* acting sequences that may be necessary for proper gene expression.

### *Transient gene expression*

After transfecting mammalian cells with foreign DNA sequences only a small proportion of cells become stably transfected with it. A significantly greater proportion of the cells may express the foreign genes for short periods of time. Such expression is referred to as transient expression. The proportion of cells expressing the foreign gene was found to be maximum at 48 h and begins to fall at 72 h. Milman and Herzberg (1981) detected the expression of transfected SV40 T-ag and the TK gene 1-3 days after addition of the DNA in 0.1-1 % of the transfected cells. If a simple *in situ* assay for the gene product is available, the transient gene expression systems provide a powerful and rapid method to screen a variety of DNA molecules to direct transcription and translation.

The levels of transient gene expression can be further improved by the use of autonomously replicating high copy number vectors such as those that contain SV40

sequences in COS cells. It may also be possible to increase the levels of transient gene expression by including an appropriate enhancer sequence on the plasmid. For example, the presence of SV40 enhancer sequences present on a plasmid containing rabbit  $\beta$ -globin sequences mediated an increased level of the globin gene expression in human cells (Banerjee *et al.*, 1981).

The rapidity with which transient gene expression assays can be conducted permit screening large numbers of DNA sequences for functionality. For example, it would be possible to test individual members of a multigene family for gene activity. It is known that all members of a multigene family need not be functional. This method has been used to identify the functionality of individual cloned members of the class I genes of the major histocompatibility genes (Barbosa *et al.*, 1982; Goodenow *et al.*, 1982; Singer *et al.*, 1982). The transient expression assays also permit determination of the strength of individual promoter sequences and effects of mutation and DNA deletion and rearrangement on gene expression.

### *Stable expression*

In cases where a selection system is utilized to isolate transfected cells, it can be inferred that the exogenous gene is expressed properly. For example, the expression of HSV TK gene in mouse cells deficient in this enzyme can be inferred by the fact that the transfected cells become resistant to medium containing HAT. Since the recipient cells were not known to revert, it was possible to infer that the HAT resistant phenotype was due to the acquisition and expression of the HSV TK gene. Similarly, cells transfected with the bacterial neo gene or gpt genes which have no cellular counterparts can be presumed to be expressing the foreign gene. But in majority of the cases it is necessary to directly assay for the transcripts and protein products.

### *Expression of non selectable genes.*

As mentioned previously, it is possible to introduce any gene sequence into mammalian cells. This is accomplished by ligated or non ligated gene transfer. In ligated transfer the non-selectable gene is covalently linked to a selectable gene and the chimera used for gene transfer. In the non ligated transfer method a large excess of the non-selectable gene is mixed with a selectable sequence in the presence of carrier DNA and presented to cells. The different DNA sequences become covalently linked in the cellular environment before integration into the cellular genome. The expression of genes introduced by either of these methods has been studied. In one study (Perucho *et al.*, 1980b), APRT, TK<sup>-</sup> L-cells were co-transformed with genomic DNA and HSV TK gene and selected for APRT<sup>+</sup> phenotype. All APRT<sup>+</sup> transformants were found to contain the TK gene sequences, as determined by blot-hybridization, and found to express the TK gene by a functional assay. In a similar study, a bacterial plasmid containing the SV40 early region was co-transformed into L-cells using the HSV TK gene as the selectable marker. Several of the TK<sup>+</sup> transformants were found to synthesize the SV40 T-antigen.

The exact nature of the transcripts produced by the transfected genes have been examined in several cases. Mantei *et al.* (1979) examined the transcription of globin

genes which were transferred by the covalent ligation method and found that most of them initiated transcription at the proper site. Roginski *et al.* (1983) examined similarly transfected human globin genes and showed that not only did the transcription initiate at the proper site but the globin mRNAs were properly polyadenylated, processed and exported to the cytoplasm. Wold *et al.* (1979) examined the expression of rabbit globin sequences introduced into mouse L-cells by the non-ligated co-transfer method. Of the six transformants analyzed, one of them showed the correct splicing pattern but 45 nucleotides that are normally present in the normal mature transcript of the  $\beta$ -globin sequences were missing. The presence of this aberrant but unique 5'-terminus suggests the possibility of incorrect initiation of the transfected gene. In a separate study a transcript 650 nucleotides longer at the 5'-terminus than the normal mature ovalbumin was detected (Breathnach *et al.*, 1980). Not all genes that are transferred by the non-ligated co-transfer method have aberrant transcription initiation, but it is possible that ligated transfer method is more reliable in yielding accurate transcription initiation and processing of non-selectable genes.

### *Regulation of gene expression*

It was found that the expression of the foreign DNA sequences after introduction into mammalian cells is regulated. The regulatory effects are dependant upon the nature of the gene as well as the nature of the recipient cell. HSV TK gene has been subjected to extensive analysis by McKnight and colleagues (McKnight *et al.*, 1981; McKnight and Kingsbury, 1982). They have shown that in addition to the TATA sequences located at -30, the CAT sequences located at -80, and additional upstream sequences are important for proper expression of the HSV TK gene.

It is now generally thought that all mammalian genes which contain the proper transcriptional signals will be transcribed in any mammalian cell. This may be true if no specialized transcriptional proteins are needed in this process. It is conceivable that such proteins may be needed for expression. Use of gene sequences which are normally expressed in specific differentiated tissues in transfection studies is needed to unambiguously answer this question.

The requirement for *trans* acting factors in properly regulated gene expression can be deduced from experiments involving the globin genes. We have shown that human  $\alpha$ - and  $\beta$ -globin genes can be readily and properly expressed in mouse L-cells (Hsiung *et al.*, 1982; Roginski *et al.*, 1983) though these genes are not regulated in these cells. Similar sequences when introduced into mouse erythroleukemia (MEL) cells not only show expression but can be induced by treatment with dimethyl sulphoxide, an agent known to induce endogenous globin gene expression in the MEL cells.

Mouse MT-1 gene is normally inducible by heavy metals including cadmium and it has been shown that this gene introduced into mouse L-cells can be induced by treatment with cadmium. Deletion mapping of this gene revealed that the minimal sequence of DNA required to elicit the heavy metal inducibility lies within 90 bp of the transcription start site (Mayo *et al.*, 1982). Corces *et al.* (1981) showed that a *Drosophila* heat shock gene can be induced in mammalian cells and the sequences necessary for this response lie within 3-6 kb of the *Drosophila* DNA segment.

Because the transfection method is relatively simple, it is possible to adapt it to assay

for regulatory sequences. The general strategy is to ligate a selectable or easily assayable gene to the putative regulatory sequences and assay the effects of such sequences on the expression of the selectable gene under various conditions. It has been shown that the expression of the p21 transforming gene of Harvey murine sarcoma virus linked to the DNA containing the LTR of MMTV is regulated by physiological concentrations of dexamethasone. Lee *et al.* (1981) observed that the MMTV sequences can drive the expression of a mouse DHFR cDNA sequence and can be regulated by glucocorticoids. All of these examples serve to illustrate the fact that gene transfection techniques permit identification of *cis* acting regulatory sequences. It is to be expected that similar methods may enable identification and isolation of genes which code for *trans* acting products which are necessary for proper regulation of genes.

#### *Gene expression affected by position of integration*

We have documented the evidence which indicates that in majority of the cases, the exogenously introduced DNA becomes integrated into the host cell DNA and that there is no site specificity for this integration. Since not all of the genome is expressed in any given cell it is to be anticipated that the sites of integration may play an important role in the expression and regulation of the transfected genes. Davidson *et al.* (1973) have described the phenomenon of phenotypic modulation in mouse L-cells transfected with UV inactivated HSV. They observed that the TK<sup>+</sup> cells lose their TK activity without losing their TK gene sequences. This phenotypic switching may occur at very low levels ( $10^{-6}$ ) or at very high levels (1–10%, Pellicer *et al.*, 1980). Clough *et al.* (1982) have shown that the low level loss of the TK<sup>+</sup> phenotype is due to hypermethylation of DNA at the CpG sequences and reexpression of the TK gene can be facilitated by treatment of the cells with 5-azacytidine, a compound which causes demethylation of mammalian cellular DNA. Observations made by Christie and Scangos (1982) and Ostrander *et al.* (1982) are also consistent with these results.

Not all of the phenotypic modulation observed in transfectants is the result of changes in methylation pattern. Davies *et al.* (1982) studied the basis for the high frequency switching of the TK phenotype in L-cells transfected with the HSV TK gene. They noted that the high frequency switching is not associated with changes in DNA methylation but with changes in chromatin structure. These results were extended by Roginski *et al.* (1983) who showed that not only TK but other adjacent sequences are also involved in the phenotypic switching.

The fate of genes introduced into mouse embryos by microinjection also support the view that the sites of integration may play an important role in gene expression. A number of investigators have introduced rabbit or human globin genes into mouse embryos (Constantini and Lacy, 1981; Lacy *et al.*, 1983; Stewart *et al.*, 1982). In all these cases the genes showed no expression or expressed at aberrant sites. Rat growth hormone genes introduced into embryos were not expressed but growth hormone gene-metallothionein gene chimeras expressed in liver and other tissues resulting in increased growth of the mice. However, additional experiments utilizing other constituent genes have to be attempted before definitive conclusions, about the factors that are important for tissue specific and developmental regulation of genes introduced into mammalian embryos, can be reached.



### Rescue and recovery of transfected genes

Transfection into mammalian cells not only permits introduction of genes but their rescue as well. The rescue and recovery methods that are used depend upon the gene that is introduced into the mammalian cells. The true eukaryotic-prokaryotic shuttle vectors can be readily moved from bacterial cells to mammalian cells and vice versa. An example of this type are the pSV2 vectors described earlier. pSV2NEO (Southern and Berg, 1982) contains a bacterial plasmid, pBR322 carrying the bacterial replication origin and the amp gene. It also contains the neo gene derived from Tn5 which can be expressed in bacterial cells or mammalian cells. This plasmid can replicate autonomously in bacterial cells and Tag<sup>+</sup> monkey cells such as COS cells (Gluzman, 1982). Purified plasmid from bacteria can be introduced into the appropriate monkey cells and after permitting their replication the low mw cellular DNA, containing the plasmid DNA sequences, can be used to transform *E. coli*. Similarly, polyoma vectors can be shuttled between mouse and bacterial cells and BPV vectors can be transferred from appropriate murine cells to bacteria.

The SV40 based vectors can be rescued from their integrated state as well. For example when pSV2 plasmids introduced into mouse or hamster cells they integrate into the host cell genome. If these cells are fused with COS cells, the Tag produced by the COS cells and specific monkey cellular factors will allow DNA replication to be initiated at the SV40 origin. Soon afterwards circular DNA molecules, containing the SV40 sequences and any plasmid or other DNA sequences surrounding them, appear in the nucleus as autonomous plasmids. These can be readily isolated by fractionating low mw DNA by a procedure described by Hirt (1967). Brietman *et al.* (1982) have shown that all of the genetic information carried on SV40 plasmid recombinants can be introduced in mammalian cells and the plasmid can be rescued by fusion with a simian cell line. They introduced the (SV40)-pBR322 recombinant vector pSV2 carrying the gpt gene of *E. coli*, into chinese hamster ovary HPRT<sup>-</sup> cells. All gpt transformed cell lines were found to contain one or more insertions of pSV2 sequence exclusively associated with high molecular weight DNA. Upon fusion with COS-1 cells, most gpt-transformed cell lines produced low-molecular weight DNA molecules related to pSV2. This rescued plasmid was tested for ampicillin resistance by transformation in *E. coli* and for gpt by transformation in mouse L-cells and gpt<sup>-</sup> *E. coli* to gpt<sup>+</sup> *E. coli*.

Hanahan *et al.* (1980) rescued a bacterial plasmid carrying the early region of SV40 (pOT) stably established in high molecular weight DNA of mouse L-cells by fusion with simian cells.

Our laboratory (Kucherlapati *et al.*, 1984) also used eukaryotic-prokaryotic shuttle vector pSV2Neo to demonstrate that mammalian somatic cells can mediate homologous recombination and the frequency of this recombination can be enhanced by pretreatment of input DNA. Two non-overlapping deletion mutants of pSV2Neo were constructed and introduced into mouse or human cells. The functional pSV2Neo plasmid was rescued from neo resistant colonies by fusion with monkey COS-1 cells which allows excision and amplification of the plasmid. Low molecular weight DNA was isolated from the cells and analyzed by propagating the plasmid in an *E. coli* strain.

Though extremely useful, it has to be noted that the shuttle vector plasmids may suffer rearrangements and mutations as a result of passaging through mammalian cells.

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The conventional method of isolating genes involves purification of the appropriate mRNA and constructing a cDNA corresponding to it and using it as a probe to screen genomic libraries. The isolation of mRNA is dependent upon its abundance in a particular cell type or knowledge about the protein product it codes for. The gene transfer system however permits isolation of genes whose products may not have been identified. The gene products should confer a selective advantage on cells carrying them or can be screened easily. Two related schemes which may be termed rescue and screening were successfully used to isolate genes coding for TK and APRT. Perucho *et al.* (1980a) used the bacterial plasmid pBR322 as a vehicle to isolate chicken thymidine kinase gene by rescue strategy. Chicken DNA was first digested with the restriction endonuclease HindIII which does not cleave the TK gene and ligated the resulting DNA to similarly digested *E. coli* plasmid pBR322 coding for ampicillin resistance. This concatenated DNA was used to transform mouse LTK<sup>-</sup> cells to TK<sup>+</sup> phenotype. Some transformants were expected to contain chicken TK gene linked to pBR322 sequences and pBR322 sequences residing in the transformants were used to rescue the flanking sequences containing TK gene. Based on this strategy, DNA from the transformant was cleaved with another restriction endonuclease which does not cleave the chicken TK gene and cleaves pBR322 only once, on either side of the single HindIII site of the circular pBR322 molecule. The cleaved DNA ligated in cyclization conditions was used to transform *E. coli* to ampicillin resistance. The rescued plasmids were tested for their ability to transfer TK back into TK<sup>-</sup> animal cells. This procedure permitted isolation of the chicken thymidine kinase gene as a 2.2 kilobase HindIII insert in pBR322.

Lowy *et al.* (1980b) adapted an alternative scheme to isolate hamster Aprt gene. This scheme does not rely on the maintenance of intact plasmid function through passage in animal cells. Hamster genomic DNA was cleaved with HindIII which leaves the Aprt gene functionally intact and was ligated to a molar excess of HindIII cleaved plasmid pBR322 DNA. This DNA was used to generate a primary Aprt<sup>+</sup> transformant which has integrated multiple plasmid sequences. The DNA from the primary transformant was transferred to recipient Aprt<sup>-</sup> cells to construct secondary Aprt<sup>+</sup> transformants containing Aprt gene linked to a single plasmid sequence. The DNA from the secondary transformants was subjected to partial cleavage with BamHI. Fractions containing 20 kbp long DNA were collected and used to construct a library of  $10^6$  independent recombinant phage using charon 4A as the vector. This library of recombinant phage was screened with highly radioactive pBR322 DNA as a hybridization probe. They screened  $6 \times 10^5$  plaques to identify a single clone containing plasmid sequences which

### Rescue and recovery of transfected genes

Transfection into mammalian cells not only permits introduction of genes but their rescue as well. The rescue and recovery methods that are used depend upon the gene that is introduced into the mammalian cells. The true eukaryotic-prokaryotic shuttle vectors can be readily moved from bacterial cells to mammalian cells and vice versa. An example of this type are the pSV2 vectors described earlier. pSV2NEO (Southern and Berg, 1982) contains a bacterial plasmid, pBR322 carrying the bacterial replication origin and the amp gene. It also contains the neo gene derived from Tn5 which can be expressed in bacterial cells or mammalian cells. This plasmid can replicate autonomously in bacterial cells and Tag<sup>+</sup> monkey cells such as COS cells (Gluzman, 1982). Purified plasmid from bacteria can be introduced into the appropriate monkey cells and after permitting their replication the low mw cellular DNA, containing the plasmid DNA sequences, can be used to transform *E. coli*. Similarly, polyoma vectors can be shuttled between mouse and bacterial cells and BPV vectors can be transferred from appropriate murine cells to bacteria.

The SV40 based vectors can be rescued from their integrated state as well. For example when pSV2 plasmids introduced into mouse or hamster cells they integrate into the host cell genome. If these cells are fused with COS cells, the Tag produced by the COS cells and specific monkey cellular factors will allow DNA replication to be initiated at the SV40 origin. Soon afterwards circular DNA molecules, containing the SV40 sequences and any plasmid or other DNA sequences surrounding them, appear in the nucleus as autonomous plasmids. These can be readily isolated by fractionating low mw DNA by a procedure described by Hirt (1967). Brietman *et al.* (1982) have shown that all of the genetic information carried on SV40 plasmid recombinants can be introduced in mammalian cells and the plasmid can be rescued by fusion with a simian cell line. They introduced the (SV40)-pBR322 recombinant vector pSV2 carrying the gpt gene of *E. coli*, into chinese hamster ovary HPRT<sup>-</sup> cells. All gpt transformed cell lines were found to contain one or more insertions of pSV2 sequence exclusively associated with high molecular weight DNA. Upon fusion with COS-1 cells, most gpt-transformed cell lines produced low-molecular weight DNA molecules related to pSV2. This rescued plasmid was tested for ampicillin resistance by transformation in *E. coli* and for gpt by transformation in mouse L-cells and gpt<sup>-</sup> *E. coli* to gpt<sup>+</sup> *E. coli*.

Hanahan *et al.* (1980) rescued a bacterial plasmid carrying the early region of SV40 (pOT) stably established in high molecular weight DNA of mouse L-cells by fusion with simian cells.

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generated  $\text{Ap}^{\text{r}+}$  colonies when added to  $\text{Ap}^{\text{r}-}$  cells with a frequency of one colony per 400 pg/ $5 \times 10^5$  cells, which was five fold greater than that obtained with total genomic DNA. Preliminary mapping data localized the functional  $\text{Ap}^{\text{r}}$  gene to an 8-kbp HindIII fragment.

Instead of using pBR322 as DNA tag, suppressor tRNA can be used as vector in the original ligation. If suppressor tRNA is used, DNA from the secondary transformant can be used to construct a genomic library with a  $\lambda$  phage vector that contains an amber mutation in the lysis gene. These phage requires a functional supF gene to complete the lytic cycle. Thus although all the DNA from the secondary transformant can be packaged, phage growth and lysis following transduction into bacteria will occur only with phage that contains supF gene which should be next to the transferred gene. The two experimental designs described should permit the isolation of any gene coding for selectable or identifiable functions for which DNA-mediated gene transfer can be used.

If human DNA is used as the donor and rodent cells are used as recipients, a DNA tag need not be used because of the presence, in the human genome, of *Alu* family of sequences (Schmid and Deininger, 1975) which are repeated several hundred thousand times. These sequences do not readily cross hybridize with any sequences in mouse cells and the probability of finding one or several *Alu* sequences near a given gene is very high. The *Alu* sequences have been used to isolate a human bladder oncogene by a screening procedure. Shih and Weinberg (1982) and Pulciani *et al.* (1982) transferred the human bladder carcinoma oncogene into mouse NIH3T3 cells. After two rounds of transfer, a genomic library was constructed in which the DNA of the transformant carried the human oncogene. The library was screened with human *Alu* sequences and the oncogene was isolated.

## Perspectives

Gene transfer methodologies have become important tools of molecular biology in a relatively short period of time. This is due to the relatively simple procedures that can be used to introduce DNA into mammalian cells and the reliable expression of these sequences in the recipient cells. The method has already proved quite useful in identification of *cis* acting elements that are needed for normal gene expression and isolation of a number of new genes whose products can be selected for in mammalian cells. Some additional problems that are currently receiving attention and has further potential are described below.

### Gene therapy

The fact that foreign genes introduced into mammalian cells can be expressed raised the possibility of conducting gene therapy. It is known that several human hereditary disorders are the result of single gene defects. In many cases, the normal counterparts of these genes have been isolated and are readily available. If these normal genes could be introduced into cells from patients who suffer from the genetic disorders, it may be possible to provide adequate amount of gene product to alleviate the effects of the disorders. Two potential methods are being tested to reach this goal. The first one

involves the use of retrovirus vectors and the second one uses homologous recombination. As described earlier, mouse ecotropic RNA viruses have been used as vectors for introduction of foreign genes into mouse cells. The development of cell lines which provide the viral coat proteins without any endogenous viral replication permit production of high titers of viruses which carry the viral LTRs and the desired gene. If such viruses are used for infection of novel cell types, the viral RNA will be converted into DNA and integrated into the cellular genome *via* the LTR. Because the LTRs contain potent promoter sequences, the inserted gene should be expressed quite efficiently. Since the viral titers are quite high it is possible to introduce the genetic information virtually into all cells in a population. Before this method can be applied to any human diseases, it is necessary to have cell lines which produce amphotropic coats which will permit introduction of the sequences into human cells. Efforts to develop such cell lines are underway.

Two potential problems of gene therapy by retrovirus mediated gene transfer are (i) the foreign sequences may integrate at a site which is needed for the normal functioning of a cell and (ii) since RNA is delivered to the cell and intervening sequences are removed before RNA is packaged into virions, it is possible to lose any *cis* acting regulatory elements that are located within the introns. The first problem may not prove to be serious because it is unlikely that very many gene insertions would result in abnormal function of the recipient cell. The second problem can be solved by introducing the desired gene in an orientation opposite to that of the viral sequences. Such a construction would prevent splicing and subsequent loss of sequences.

An alternative to using viral vectors for gene therapy is to replace the resident gene with the exogenous gene by a homologous recombination mechanism. It is now clear that somatic mammalian cells are capable of mediating homologous recombination between two exogenously introduced plasmids at very high efficiencies. Several investigators are exploiting this observation to determine if an exogenously introduced plasmid containing a sequence homologous to a cellular gene can recombine in a homologous fashion with the cellular gene. If this is successful and occur at appreciable frequencies, it ought to be possible to obtain gene replacement without altering or affecting any other gene in the genome.

#### *Study of development and differentiation.*

The processes of cellular differentiation and development can be considered as a series of steps of gene activation and inactivation. It is believed that these series of steps are mediated by transacting factors acting on DNA sequences of specific genes. If this were the case, gene transfer methods offer a way to identify such sequences. Once identified, *in vitro* modification of such DNA segments and reintroduction into the animal embryo should elicit responses which may be correlated with specific alterations. This strategy assumes that the exogenously introduced sequence would show normal developmental regulation. Though there are no current reports of such regulation, it is anticipated that examples of this nature would be forthcoming in the immediate future.

An alternative possibility offered by the gene transfer system is that an exogenous DNA segment would integrate at a locus which is important for developmental regulation resulting in a recessive lethal phenotype. If this were the case the exogenous

DNA segment can be used as a probe to isolate the developmentally important gene. Indeed Jaenisch *et al.* (1983) recently reported that a retrovirus sequence introduced into the mouse embryo resulted in a developmental lethal mutation. They have been able to identify this locus to be that coding for collagen (Harbers *et al.*, 1984). A similar observation of possible gene inactivation through insertional mutagenesis was reported by Wagner *et al.* (1983).

The general strategy of insertional mutagenesis can be applied not only to study development and differentiation of embryos but also of cultured cells. The only requirement is that the loss of a phenotype can be readily detected or selected.

### *Mechanisms of mutagenesis*

Though a number of agents are known to be mutagenic to mammalian organisms or cells, the exact mechanism by which they cause mutations or their specificity is not well understood. Gene transfer systems may offer a method to study these processes. The feasibility of one strategy has been tested for this purpose. It involved the introduction of a shuttle vectors carrying two genes into a mammalian cell, permit it to undergo several rounds of DNA replication, rescue it in bacteria and examine it for mutations and structural alterations. Unfortunately experimental results from two different laboratories revealed that the shuttle vector plasmids have a tendency to undergo spontaneous mutations and rearrangements at a rather high frequency (Calos *et al.*, 1983; Razzague *et al.*, 1983, 1984). However, it is possible that different types of vectors and recipient cells may alleviate this problem. Alternatively, it may be possible to introduce a mutation into a specific site of a specific gene, introduce it into mammalian cells and determine if it is possible to induce its reversion by specific mutagenic agents.

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## How viruses damage cells: alterations in plasma membrane function

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**Abstract.** The effect of viruses on plasma membrane function has been studied in two types of situation: (i) during the toxin-like action of paramyxoviruses when fusing with susceptible cells, and (ii) during an infectious cycle initiated by different viruses in various cell types.

The nature of the permeability changes induced during the toxin-like action of viruses, and its modulation by extra-cellular  $\text{Ca}^{2+}$ , are described: membrane potential collapses, intracellular ions and metabolites leak out of, and extracellular ions leak into cells, but lysis does not take place. The biological significance of such changes, and their relation to changes induced by other pore-forming agents, are discussed.

Changes in membrane permeability such as those mentioned above have not been detected during infection of cultured cells by paramyxo (Sendai, measles, mumps), orthomyxo (influenza), rhabdo (vesicular stomatitis), toga (Semliki Forest) or herpes viruses. On the contrary, sugar uptake is increased when BHK cells are infected with vesicular stomatitis virus, semliki forest virus or herpes virus. Cultured neurones infected with herpes simplex virus show changes in electrical activity. The pathophysiological significance of these alterations in membrane function, which occur in viable cells, is discussed.

It is concluded that clinical symptoms may result from cell damage caused by virally induced alterations of plasma membrane function in otherwise intact cells.

**Keywords.**  $\text{Ca}^{2+}$ ; permeability; plasma membrane; viruses.

### Introduction

The plasma membrane is the first part of a cell with which a virus comes in contact when invading tissues and much of the host cell specificity of viral action is due to binding between particular components at the cell surface and proteins on the surface of the virus. Entry of the viral genome which follows, is generally by endocytosis of the intact virus; the genome is released into the cytoplasm following breakage of the endocytic membrane. In the case of paramyxoviruses, a family of enveloped RNA viruses that contain a glycoprotein in their surface capable of initiating membrane fusion at neutral pH, the viral genome is introduced directly into the cell as a result of fusion between the viral envelope and the cell plasma membrane (figure 1).

The plasma membrane is usually involved a second time following uncoating and expression of the viral genome inside the cell. In the case of enveloped viruses that are released by budding from the plasma membrane, proteins of the viral envelope become inserted into the membrane prior to budding. For other viruses also, virus-coded

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Abbreviations used: BHK, Baby hamster kidney; dGlc, deoxyglucose; VSV, vesicular stomatitis virus; SFV, Semliki Forest virus; HSV, herpes simplex virus; MeGlc, methyl glucose.

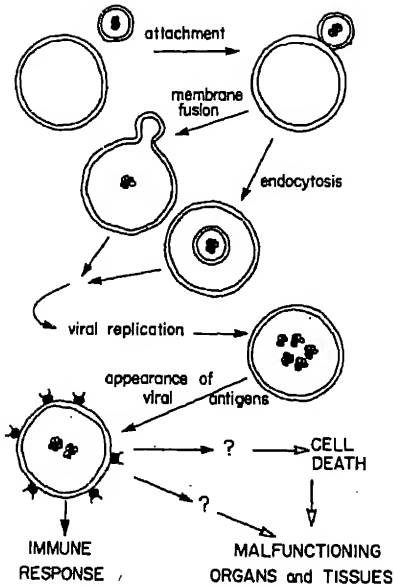


Figure 1. Events during the infection of cells by viruses.

proteins appear at the cell surface and this forms the basis of the immune response to viral infection. Moreover, new host-coded proteins may appear at the cell surface, and existing proteins become modified, with or without triggering an immune response; the transforming viruses are an example of this situation.

During acute infections by cytolytic viruses, especially by the non-enveloped ones, in which the infected cell finally bursts as large amounts of virus are released, the plasma membrane becomes damaged to the point of rupture. At this time, cells become permeable to trypan blue, to cytoplasmic proteins which leak out, and to ions such as  $\text{Na}^+$  and  $\text{K}^+$  (the capacity of the  $\text{Na}^+$  pump to reverse this trend having been exceeded). The latter event leads to the entry of water and to cell swelling, and this is a contributing factor to the lytic action of these viruses.

In terms of the pathophysiological consequences of viral action it is generally assumed that cell lysis, leading to tissue necrosis, is the underlying cause (figure 2). Yet there is no more reason for assuming that cell death is necessarily responsible for the symptoms of viral infection, than there is in the case of diseases such as juvenile diabetes or the haemoglobinopathies: in these cases the symptoms of the disease are due to an alteration in the function of muscle or blood cells respectively, not to their destruction. Such considerations have led the author to pose the question that forms the basis of this article: to what extent do virally-mediated alterations of plasma membrane function

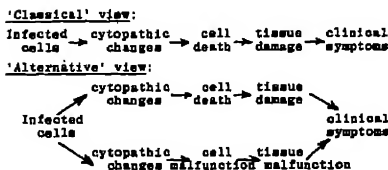


Figure 2. Pathogenesis of viral disease.

underlie changes in cell behaviour? We have examined two situations: the first is that of viral entry; the second is that of alterations during the early stages of the infectious process (which may bear similarity to the case of a persistent infection). The third situation, that of events just prior to cell lysis, is pertinent to the induction of cell death, rather than to that of cell damage (figure 2), and will not be discussed further. Several authors have not distinguished sufficiently critically between the second and third situation: a partial leakage of ions, for example, has been interpreted as being distinct from cell lysis (Carrasco and Lacal, 1984); yet this may reflect no more than the fact that lysis of cells is not synchronous, so that an apparent partial leakage of ions is actually due to complete leakage (lysis) from some cells, and no leakage from others.

### Viruses as toxins

Toxins may be defined as substances that damage cells *in vitro*, and as a result cause disease *in vivo*. Certain constituents of the venom of wasps, spiders or snakes are examples, as are the proteins produced by gram negative and gram positive bacteria (figure 3). In the case of viruses, the postulate is that the viruses themselves are the toxins. Just as cholera, diphtheria or botulism results from an interaction between bacterial proteins and cells without the occurrence of an infectious cycle (figure 3), so it is suggested that clinical symptoms result from interaction between certain viruses and susceptible cells without the participation of an infectious cycle (figure 4). Haemolytic paramyxoviruses are obvious candidates for study. This is because during the entry process, achieved by fusion between viral envelope and cell plasma membrane, the cell becomes leaky to the extent that, in erythrocytes, lysis (*i.e.* haemolysis) ensues. Since lysed cells are no longer viable, the situation is one of cell death, not cell damage. The reason for discussing haemolytic paramyxoviruses in the present context is that in cells other than erythrocytes, lysis is often not the end result, and instances will be described in which changes in cell permeability lead to a transient alteration of cell behaviour.

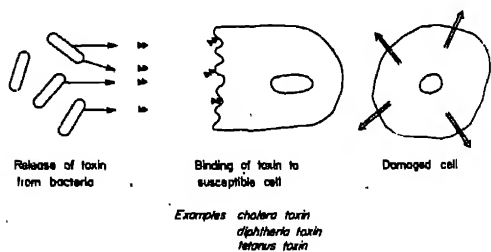


Figure 3. Action of bacterial toxins.

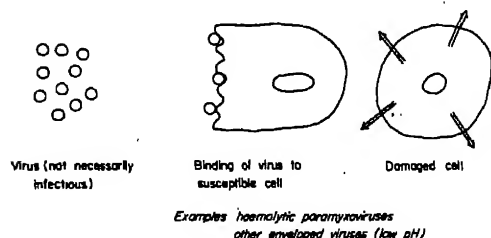


Figure 4. Toxin-like action of viruses.

*Permeability changes induced by haemolytic paramyxoviruses*

The system studied most extensively to date is that of Sendai virus interacting with Lettrec cells (a line of malignant mouse ascites cells akin to Ehrlich ascites cells). Of other viruses tested so far, only Newcastle Disease virus acts in a similar way (Poste and Pasternak, 1978; Foster *et al.*, 1980), though other enveloped viruses (such as influenza) do so if the pH is reduced to below 6 (Patel and Pasternak, 1983). The specificity with regard to cell type is much less, and every cell so far tested (except sheep erythrocytes; unpublished observation) responds to Sendai virus (Pasternak, 1984); this is because the requirement for viral binding is no more specific than the presence of a sialic acid-containing glycoprotein (Scheid, 1981), and most cells possess such molecules at their surface.

The events that occur when virus is added to cells are summarized in figure 5. Binding between virus and cells is a temperature-independent process. It is followed by fusion between the viral envelope and the cell plasma membrane; membrane fusion is a highly temperature-dependent process, with a  $Q_{10}$  of approximately 7 between 7 and 37°C (Micklem *et al.*, 1984a). Membrane fusion commences without a lag (Micklem *et al.*, 1984a), though the onset of permeability changes is characterized by a temperature-dependent lag period (Pasternak and Micklem, 1973; Micklem and Pasternak, 1977); lag appears to reflect the build-up of a sufficient amount of membrane damage to be manifest as changes in properties such as surface membrane potential (Okada *et al.*, 1975; Fuchs *et al.*, 1978; Impraim *et al.*, 1980; Bashford *et al.*, 1983a,b), permeability of monovalent (Fuchs and Giberman, 1973; Pasternak and Micklem, 1974a; Poste and Pasternak, 1978; Bashford *et al.*, 1983a, b) and divalent (Getz *et al.*, 1979; Impraim *et al.*, 1979; Fuchs *et al.*, 1980; Hallett *et al.*, 1982) cations, permeability of phosphorylated low molecular weight metabolites such as phosphoryl choline (Pasternak and Micklem, 1973), sugar phosphates (Pasternak and Micklem, 1973), nucleotides (Impraim *et al.*, 1980), low molecular weight peptides (Wyke *et al.*, 1980), and so forth. The cut-off point appears to be at a molecular weight of approximately 1000, so that proteins and other macromolecules do not leak across cells (Pasternak and Micklem, 1973; Poste and Pasternak, 1978), except at high doses of virus (Tanaka *et al.*, 1975; Yamaizumi *et al.*, 1979). That is why these viruses, though haemolytic, are not cytolytic (Knutton *et al.*, 1976). Lag, that is the acquisition of a threshold number, or size, of permeability pores is shorter for membrane depolarization than for leakage of ions, which is shorter than for leakage of phosphorylated metabolites; thus the onset of permeability changes is sequential (figure 6). Since cells recover from the effects of virus, through membrane turnover, through lateral dispersal of protein 'pores', and through other mechanisms, it

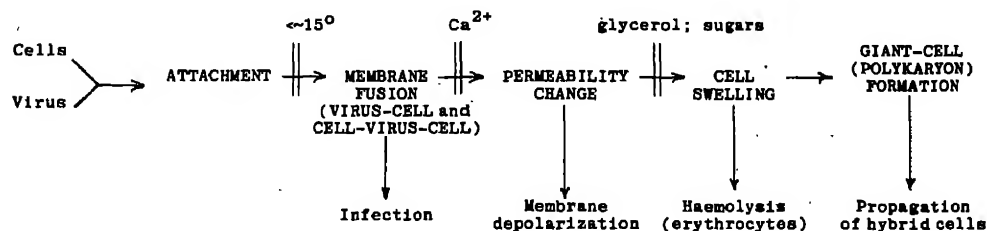


Figure 5. Events induced by haemolytic paramyxoviruses.

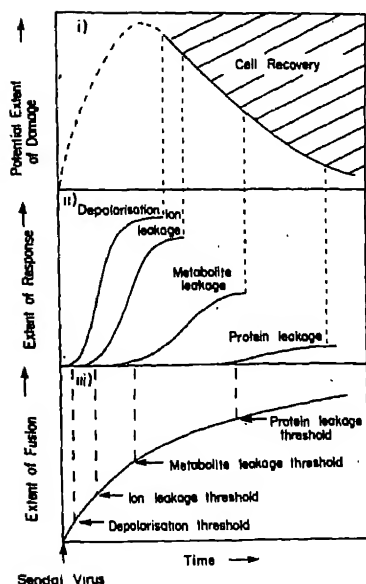


Figure 6. Sequential onset of permeability changes.

is possible under certain conditions to observe membrane depolarization without ever initiating leakage of phosphorylated metabolites, for example (Pasternak, 1984).

#### *Modulation of permeability changes: $\text{Ca}^{2+}$ and $\text{Ca}^{2+}$ antagonists.*

Some 30 years ago it was noted that haemolysis by Newcastle Disease virus (Burnet, 1949), mumps virus (Morgan, 1951) and Sendai virus (Fukai and Suzuki, 1955) is prevented by high concentrations of extracellular  $\text{Ca}^{2+}$ . Since then, the inhibitory effect of  $\text{Ca}^{2+}$  at physiological concentrations (*i.e.* mM) on Sendai virus-mediated permeability changes has been demonstrated in a number of systems (Pasternak and Micklem, 1974a; Pasternak *et al.*, 1976; Impraim *et al.*, 1979; Foster *et al.*, 1980; Forda *et al.*, 1982).  $\text{Ca}^{2+}$  exerts its action in several ways, all of which may be the result of an interaction with one particular type of receptor at the cell surface: the lag period to onset of permeability changes is lengthened (*i.e.* threshold is increased), the leakage of ions and metabolites across the membrane is partially inhibited, and recovery of cells is accelerated (Impraim *et al.*, 1980). Although the amount of  $\text{Ca}^{2+}$  required to prevent permeability changes is proportional to the amount of virus added,  $\text{Ca}^{2+}$  acts neither to prevent virus-cell binding (Wyke *et al.*, 1980), nor to prevent virus-cell fusion (Pasternak, 1981). How it does act remains to be established: while there is a similarity with communicating junctions in that, these too, are blocked by  $\text{Ca}^{2+}$ , the concentration of  $\text{Ca}^{2+}$  required in that case is in the micromolar (Unwin and Ennis, 1984), not millimolar range, since it acts intracellularly (Rose and Loewenstein, 1975); moreover  $\text{Mn}^{2+}$  is as effective as  $\text{Ca}^{2+}$  in blocking virally induced permeability changes, [though  $\text{Mg}^{2+}$  is not (Impraim *et al.*, 1979)] whereas  $\text{Mn}^{2+}$  is ineffective at closing communicating junctions.

In order to try and understand the mechanism by which  $\text{Ca}^{2+}$  affects permeability changes, we have examined the effect of a number of drugs known to block the action of



$\text{Ca}^{2+}$  in excitable cells (Fleckenstein, 1977; Henry, 1980). Surprisingly, these drugs proved to have an anti- $\text{Ca}^{2+}$ -like action in Lettre cells also. Thus verapamil, D600 and prenylamine shorten lag, and increase the extent of leakage; in the presence of a concentration of  $\text{Ca}^{2+}$  which on its own would inhibit leakage entirely, the addition of prenylamine allows leakage of ions and phosphorylated metabolites to occur. The drugs do not affect leakage itself, in contrast to  $\text{Ca}^{2+}$  chelators such as EGTA. Other types of compound which we tested are inhibitors of calmodulin, such as trifluoperazine and calmidazolium (R24571): these proved to have an action similar to the  $\text{Ca}^{2+}$  antagonists; they are active at the same concentrations ( $10^{-5}$  M trifluoperazine and  $10^{-6}$  M calmidazolium) as those at which they inhibit calmodulin-activated enzymes such as phosphodiesterase (Van Belle, 1981). It has been postulated that  $\text{Ca}^{2+}$  antagonists such as verapamil, D600 and prenylamine bind to calmodulin at the same hydrophobic site as trifluoperazine and calmidazolium (Johnson and Wittenauer, 1983). When the binding constants of all these drugs were compared with the concentrations of drug required to achieve 50% stimulation of leakage, the two sets of data were found to be in reasonable agreement (Micklem *et al.*, 1984b), suggestive of a role of calmodulin in protecting the cell surface against virally-mediated permeability changes (figure 7).

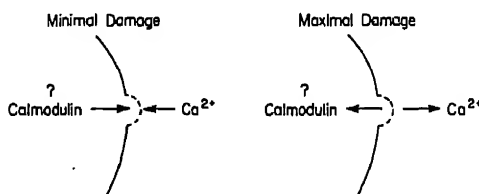


Figure 7. Role of  $\text{Ca}^{2+}$  in protecting against virally-mediated permeability changes.

The broken line indicates a virally-induced pore;  $\text{Ca}^{2+}$  (a) prevents the induction of pores, (b) inhibits leakage through pores and (c) accelerates dispersal of pores ('recovery'). Direct evidence for the participation of calmodulin has not yet been obtained; the reason for implicating it is merely that inhibitors of calmodulin have an anti- $\text{Ca}^{2+}$  like action.

From Micklem *et al.* (1984b).

A word of caution is, however, necessary. First, a detergent like triton X100 has an action, at a concentration well below its critical micellar concentration, similar to the  $\text{Ca}^{2+}$ -antagonist drugs; it should be emphasized that all the agents mentioned affect permeability changes at a concentration at which they are without effect in the absence of virus. While there is no reason why an aromatic compound such as triton should not bind to the same site as the (aromatic)  $\text{Ca}^{2+}$ -antagonists, the same might not be expected of a non-aromatic detergent like lubrol; yet lubrol is as effective as triton X100 (unpublished experiment suggested by Dr Carlos Gitler). Second, the  $\text{Ca}^{2+}$  antagonists are much more effective at stimulating permeability changes at low temperature (*e.g.*  $20^{\circ}\text{C}$ ), than at  $37^{\circ}\text{C}$ . This suggests that the drugs might affect membrane fusion (see above). On the other hand fusion is not a  $\text{Ca}^{2+}$ -sensitive event; moreover the induction of permeability changes by purified preparations of the bee venom protein mellitin (see below) is also susceptible to stimulation by calmidazolium at low temperature. Since membrane fusion does not occur in this instance the drugs are more likely to have a

general 'membrane-weakening' effect. Indeed, there is accumulating evidence that the action of calmodulin inhibitors is not as specific as previously thought (Landry *et al.*, 1981; Corps *et al.*, 1982; Gomperts, 1984). Hence it is premature to draw any conclusions other than that  $\text{Ca}^{2+}$  protects cells against virally-induced permeability changes, and that detergents and drugs that happen to have an anti- $\text{Ca}^{2+}$ -like action potentiate permeability changes.

### *Similarity to other pore-forming agents*

If the action of haemolytic paramyxoviruses on cells is due to the induction of some kind of hydrophilic pore in the plasma membrane, then the sequence of events described above,—namely membrane depolarization, leakage of ions, leakage of phosphorylated metabolites,—is likely to occur during the generation of pores by other toxin-like substances also. We have examined three types of pore-forming agents, and each exhibits just such a response at sub-lytic concentration: the protein mellitin of bee venom (Haberman, 1972), the  $\alpha$ -toxin of *Staphylococcus aureus* (Arbuthnott, 1982) and the factors of activated complement (Mayer, 1972; reviewed by Bhakdi and Trandum-Jensen, 1983, 1984 and by Muller-Eberhard, 1984). In each case extracellular  $\text{Ca}^{2+}$  inhibits the response; in no case, of course, is membrane fusion involved. Higher concentrations of  $\text{Ca}^{2+}$  are required to prevent permeability changes, especially those induced by *S. aureus*  $\alpha$ -toxin, than by Sendai virus, and in the case of  $\alpha$ -toxin,  $\text{Mg}^{2+}$  is as effective as  $\text{Ca}^{2+}$  (Bashford *et al.*, 1984a). Sensitivity to inhibition of pore-formation by divalent cations clearly depends both on the cell type under study (Lettre cells are more sensitive than erythrocytes, for example) and on the nature of the agent used. The latter observation is hardly surprisingly in view of the fact that the effective pore size varies, from approximately 1 nm diameter (Sendai virus; Wyke *et al.*, 1980), to 2–3 nm ( $\alpha$ -toxin; Fussle *et al.*, 1981) to 0.9–7 nm (complement, depending on the amount of C9; Bhakdi and Trandum-Jensen, 1984). Despite such differences the overall similarity in response to extracellular  $\text{Ca}^{2+}$  makes study of these pore-forming agents in relation to the toxin-like action of viruses worthwhile.

### *Biological significance of permeability changes*

A change in the permeability properties of excitable cells is likely to affect their function. It is not surprising, therefore, that Sendai virus causes cultured neurones to lose excitability, cultured heart cells to stop beating, and isolated pituitary cells to secrete ACTH and other hormones (Forda *et al.*, 1982; Pasternak and Micklem, 1984). The effect is presumably caused by membrane depolarization and/or  $\text{Ca}^{2+}$  entry. Such experiments have to be conducted at sub-physiological concentrations of  $\text{Ca}^{2+}$ ; at higher concentrations of  $\text{Ca}^{2+}$  the effects are abolished (see above). The interesting point is (a) the rapidity of the response (a change in membrane potential of neurones within seconds of adding virus) and (b) the transient nature of the response (complete restoration of neuronal excitability, of heart beat, or of cessation of hormone release, within minutes). In short, the physiological function of the respective cells is impaired without loss of viability (see figure 2), and with recovery of the original function. Such situations provide clear illustrations of how the toxin-like action of viruses can lead to a

transient alteration of cell behaviour. To what extent they contribute to clinical symptoms during viral infections is an important topic for future investigations.

One area where virally-induced permeability changes might play a role is in the effect of viruses on neutrophils and macrophages. An impairment of neutrophil function, for example, has been suggested to be responsible for the secondary bacterial infections that often follow a primary infection of the respiratory tract by viruses such as influenza (Larson and Blades, 1976). Using luminol-induced chemiluminescence as an indicator of neutrophil function (namely the generation of oxygen radicals within cells), however, we have found that permeability changes underly neither the action of influenza virus, nor that of Sendai virus, on neutrophils (S. Mehta and C. A. Pasternak, unpublished results).

Virally induced permeability changes also have a role in biological research. First, the use of Sendai virus to mediate cell-cell fusion and the generation of hybrid cells (Harris, 1970) depends on cell swelling brought about by the increased permeability to ions (Knutton and Pasternak, 1979). Second, the generation of a permeability pore of approximately 1 nm allows the introduction into cells of molecules of < 1000 daltons such as cyclic nucleotides, EGTA and similar compounds that bind  $\text{Ca}^{2+}$ , etc. The latter type of agent has been used to introduce  $\text{Ca}^{2+}$  at micromolar concentrations into mast cells (Gomperts *et al.*, 1981, 1983) and pituitary cells (Gillies *et al.*, 1981; Pasternak, 1984), in order to assess what concentration of  $\text{Ca}^{2+}$  is required intracellularly in order to trigger histamine release and ACTH release, respectively, from these cells. The fact that virally-induced permeability changes are transient, and can be controlled by extracellular  $\text{Ca}^{2+}$ , makes this method of permeabilizing cells preferable to techniques employing detergents (Miller *et al.*, 1979) or electric shock (Baker and Knight, 1978).

### Surface changes during viral infection

We have measured three surface properties during the course of an infectious cycle initiated by a number of different viruses: permeability of various cells to ions and low molecular weight compounds, sugar transport by baby hamster kidney (BHK) cells and excitability of cultured neurones.

The reason for the first investigation is obvious. Having demonstrated that certain paramyxoviruses induce a permeability change when entering cells by membrane fusion, it was of interest to examine whether similar changes occur when these and other enveloped viruses are released from cells at the end of an infectious cycle by what is essentially the reverse process, namely 'budding' from the cell surface. Moreover it has been suggested not only that permeability changes do occur during the maturation of various viruses (Carrasco, 1977, 1978; Carrasco and Lacal, 1984), but that such changes underly the mechanism by which cells switch from the synthesis of host proteins to that of viral proteins (Carrasco and Smith, 1976, 1980) (figure 8).

The second investigation arose from the first. We were using [ $^3\text{H}$ ]-deoxyglucose (dGlc) uptake by BHK cells as a measure of an increased permeability to low molecular weight compounds such as sugar phosphates, and found an unexpected result: instead of taking up less [ $^3\text{H}$ ] (as cells permeabilized with Sendai virus, for example, do, since [ $^3\text{H}$ ]-dGlc-6-P formed intracellularly immediately leaks out again; Imprim *et al.*,

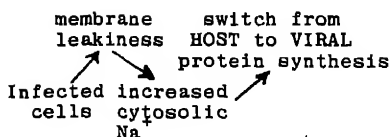


Figure 8. Hypothesis of Carrasco and Smith (1976, 1980).

1980), BHK cells infected with a number of different viruses were found to take up more [ $^3\text{H}$ ]. The basis for this apparently anomalous effect has now been explored in some detail.

The third investigation was triggered by the fact that herpes viruses are known to infect ganglionic neurones and, in the case of a herpes virus such as *Varicella zoster* (chicken pox virus), may remain latent there for many years; a study of the electrical properties of the cell surface of herpes simplex virus (HSV)-infected neurones yielded another unexpected result, and this system was therefore investigated further.

### Membrane permeability

In contrast to the permeability changes induced by the toxin-like action of paramyxoviruses, changes induced during viral budding from cells, or at some earlier stage of the infectious cycle, are likely to be more stable, and to appear at some characteristic time after the establishment of the viral genome within the cell (figure 9). Infection of various cell types, grown in monolayer or in suspension culture, with myxoviruses such as Sendai, measles, mumps or influenza virus (Foster *et al.*, 1983), with a rhabdovirus (vesicular stomatitis virus, VSV), a togavirus (Semliki Forest virus, SFV) (Gray *et al.*, 1983a) or a herpes virus (HSV) (M. H. James and C. A. Pasternak, unpublished results), have not revealed any permeability changes similar to those described in the first part of this article. True, there does appear to be a decreased concentrative uptake of sugars and amino acids (comparable with an increase in membrane permeability; Pasternak

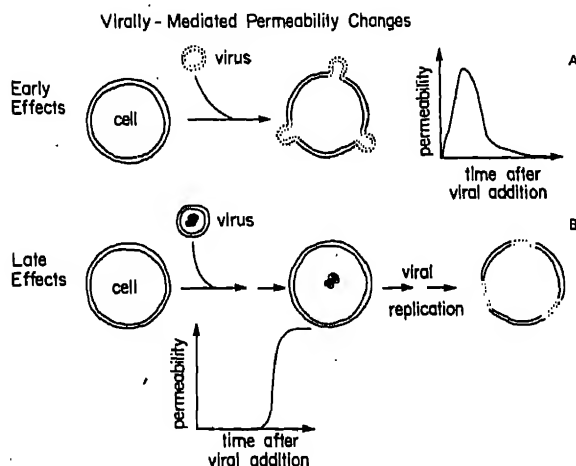


Figure 9. Permeability changes induced by viruses.

A. Toxin-like changes. B. Changes during an infectious cycle.

and Micklem, 1974b; Impraim *et al.*, 1980) by certain cells infected in suspension culture (Pasternak and Micklem, 1981), but the effect is much smaller than that induced by the toxin-like action of haemolytic paramyxoviruses; moreover the decreased uptake of nutrients is not accompanied by leakage of monovalent cations down their concentration gradients (Pasternak *et al.*, 1982; Gray *et al.*, 1983a; Foster *et al.*, 1983), as would be expected if the permeability barrier of the cell had been breached by the creation of a hydrophilic pore. And although the membrane potential of some cells appears to decrease after infection with certain viruses (Pasternak and Micklem, 1981; Pasternak *et al.*, 1982; Bashford *et al.*, 1984b), this is not the result of a generalized increase in membrane permeability.

In one instance, namely in SFV-infected BHK cells, a modest increase in intracellular  $\text{Na}^+$  does occur, though without any decrease in intracellular  $\text{K}^+$  (Gray *et al.*, 1983a). Since it is an increase in intracellular  $\text{Na}^+$  that has been postulated to account for the shut-down of host protein synthesis (Carrasco and Smith, 1976, 1980; Garry and Waite, 1979; Garry *et al.*, 1979, 1982), this situation was examined further. It was found that a greater increase in intracellular  $\text{Na}^+$ , brought about either by the addition of the  $\text{Na}^+/\text{K}^+$  ionophore nigericin or by a brief exposure of cells to haemolytic Sendai virus, resulted in a much lesser effect on protein synthesis than did infection with SFV (Gray *et al.*, 1983b). Hence even in this case an altered intracellular  $\text{Na}^+$  concentration (whether achieved as a result of a permeability change or not) cannot adequately account for the shut-down of host protein synthesis.

Another aspect of Carrasco's hypothesis (figure 8), that infected cells become permeable to low molecular weight compounds (Carrasco, 1977), is the following. Infected cells might be expected to take up low molecular weight inhibitors of protein synthesis, such as the GTP analogue GppCH<sub>2</sub>p, better than uninfected cells, and as a result of impaired protein synthesis become so incapacitated as to stop viral production. This is precisely what was found in 3T6 and BHK cells infected with EMC, SFV or mengo virus (Carrasco, 1978). When we measured the uptake of [<sup>3</sup>H]-GppCH<sub>2</sub>p by BHK cells, however, we found no difference between SFV-infected and uninfected cells, despite the fact that protein synthesis was indeed inhibited more strongly by GppCH<sub>2</sub>p in infected, than in uninfected cells. This apparent anomaly was partially resolved when it was found, in confirmation of an earlier report (Whitehead *et al.*, 1981), that infected cells have a lower GTP/GDP ratio than uninfected cells, making the GTP analogue GppCH<sub>2</sub>p a relatively more potent inhibitor of protein synthesis (Gray *et al.*, 1983c). Hence the greater efficacy of GppCH<sub>2</sub>p is due not to an increased intracellular concentration of GppCH<sub>2</sub>p, but to an impaired metabolism in infected cells. Indeed, SFV-infected BHK cells become sensitive to inhibition of protein synthesis by GppCH<sub>2</sub>p after their rate of protein synthesis has already begun to decline (Gray and Pasternak, 1984).

Other inhibitors of protein synthesis, such as the antibiotic gougerotin, have been shown to have an effect similar to GppCH<sub>2</sub>p in inhibiting protein synthesis in virally-infected cells. We have found no difference in uptake of [<sup>3</sup>H]-gougerotin by infected as compared with uninfected BHK cells (M. A. Gray and C. A. Pasternak, unpublished results). From this, and the other results described in this section, it must be concluded that, whatever the reason for the shut-down of protein synthesis in virally-infected cells, it is not the result of an increased permeability at the plasma membrane.

### Sugar transport

The observation that BHK cells infected with VSV, SFV (Gray *et al.*, 1983a) or HSV (M. H. James and C. A. Pasternak, unpublished results) are able to take up 2-dGlc 2–3 times better than uninfected cells has led us to an investigation of sugar transport in infected cells. Glucose uptake is difficult to measure (because of its rapid conversion to most cell constituents as well as to CO<sub>2</sub>) and analogues of glucose are therefore routinely used instead. dGlc is taken up by the glucose transport system, and subsequently phosphorylated by hexokinase; it is not further metabolized to an appreciable extent, and accumulation of dGlc-6-P in cells can be used as a measure of the rate of uptake of dGlc (Wohlheuter and Plageman, 1980). 3-O-Methyl glucose (3-MeGlc) is also taken up by the glucose transport system, but it is not a substrate for hexokinase.  $\alpha$ -Methyl glucoside ( $\alpha$ -MeGlc) is neither a substrate for the glucose transport system nor for hexokinase (it enters cells by simple passive diffusion only). Using radiolabelled dGlc, 3-MeGlc and  $\alpha$ -MeGlc, we have obtained the results indicated in table 1. From this it is clear that it is sugar transport, not the subsequent metabolism, that is stimulated in infected cells. Preliminary experiments suggest that the  $V_{\max}$  for transport is unaffected, and that the effect is therefore on  $K_m$ . Attempts to measure the number of Glc transporter sites by binding of [<sup>3</sup>H]-cytochalasin B (Baldwin and Baldwin, 1981) do not indicate an increased number of sites in infected cells, which is in keeping with the lack of effect on  $V_{\max}$ .

Table 1. Effect of viral infection on sugar uptake by BHK cells.

Sugar	Facilitated uptake	Phosphorylation	Further metabolism	Uptake increased in infected cells
Glc	+	+	+	ND
dGlc	+	+	—	+
3-MeGlc	+	—	—	+
$\alpha$ -MeGlc	—	—	—	—

Pooled data for BHK cells infected with SFV or HSV (unpublished results of M. A. Gray and M. H. James).

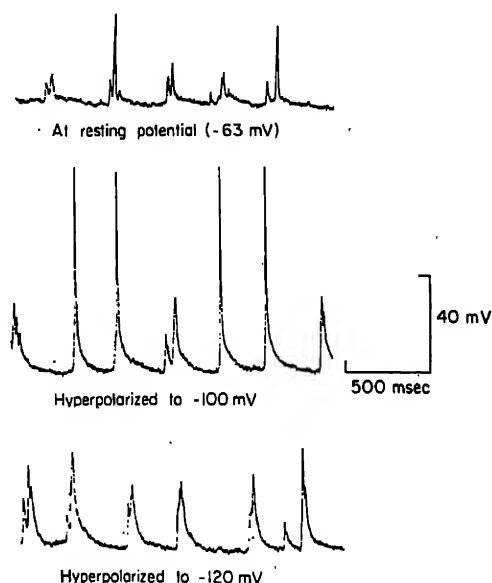
It is somewhat surprising that infection of BHK cells by viruses such as VSV, SFV or HSV, which in each case eventually leads to cytolysis of the cells, should induce an increased uptake of sugar. For such an increase has to date been associated with transformation of cells by oncogenic viruses (reviewed by Pasternak and Knox, 1979), or with the progression of non-malignant cells to malignancy (White *et al.*, 1981, 1983); in these cases DNA, RNA and protein synthesis are stimulated, whereas in infected cells the opposite is the case. Clearly an increased sugar uptake is symptomatic of some other alteration of cell metabolism. A clue as to what this might be has come from the use of temperature-sensitive mutants of HSV. In one such mutant (K), which is blocked at a very early stage in the infectious cycle, increased sugar uptake is also blocked, whereas in other mutants that are blocked at later stages, increased sugar uptake is unaffected. The gene affected in mutant K expresses a protein that, among other things, appears to be involved in the synthesis of stress proteins; these are proteins that are synthesized

when cells are subjected to various forms of stress, such as a heat shock, viral infection, the presence of certain chemicals, etc. The function of stress proteins is unknown: the present results suggest that one function may be related to an increase in sugar uptake.

### Neuronal excitability

If the dorsal root ganglia in the spinal cord of embryonic chicks or neonatal rats are dissected out and cultured in the presence of inhibitors of cell division, fairly pure preparations of single neurones can be obtained; impalement with stimulating and recording electrodes can then be used to measure electrophysiological parameters such as excitability (*i.e.* the generation of an action potential). This is the system that was used to show the rapid and transient toxin-like effect produced by Sendai virus (Forda *et al.*, 1982; Pasternak and Micklem, 1983) that was referred to in a previous section.

When such neuronal cells are infected with HSV, there is no discernible change in electrophysiological properties for some hours. About 8–10 h after infection, when viral antigens begin to detectable, one or two changes occur (James and Mayer, 1984). With one particular strain of HSV excitability declines, apparently due to the loss of 'fast'  $\text{Na}^+$  channels, as previously noted by others (Fukada and Kurata, 1981). With another strain excitability is unaltered, but the threshold is reduced to the point that cells begin to fire action potentials spontaneously, *i.e.* without any applied stimulus. Such activity, which is depicted in figure 10, has not previously been recorded; [during



**Figure 10.** Spontaneous electrical activity in Herpes-infected neurones.

Rat dorsal root ganglionic neurones in culture were infected with HSV1 and electrical recordings made as described in Forda *et al.* (1982). The trace shows the electrical activity of neurones 15 h after infection; uninfected cells show no spontaneous activity.

Unpublished experiments of M. H. James and M. L. Mayer.

the preparation of this manuscript, a preliminary communication appeared (Lima *et al.*, 1983) that describes essentially similar results]. Since the neurones under study are probably sensory neurones, an intriguing possibility is raised: namely that what is being measured *in vitro* is in some measure related to the pain experienced by patients harbouring certain types of herpes virus. Clearly a more detailed study of this system, which is another example of the effects of a viral infection on the function of cells without killing them (figure 2), is likely to be rewarding in terms of mechanism and therapeutic approaches pertinent to the pathophysiology of infections caused by herpes viruses.

## Conclusions

This article has described some of the alterations in plasma membrane function that are induced by viruses. Although we have restricted ourselves to rather simple systems, it is clear that viruses are able to damage cells without lysis; indeed in the case of the toxin-like action of certain paramyxoviruses, in the absence of an infectious cycle at all. The idea that a viral infection can affect cell behaviour without cell death or even any obvious cytological change has recently been raised by other groups as well (Fields, 1984) and a particularly clear-cut case has been presented for the effects of lymphocytic choriomeningitis virus on pituitary function (Oldstone *et al.*, 1984). This is clearly an area for fruitful research in the future.

The proposal that some of the actions of certain viruses should be considered as toxin-like, which has been made by others also (Smith, 1983), leads one to compare the effects of, for example, haemolytic paramyxoviruses with those of haemolytic bacterial toxins like *S. aureus*  $\alpha$ -toxin. Each agent induces hydrophilic pores through which ions can move so freely that the capacity of the  $\text{Na}^+$  pump to exclude  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{H}_2\text{O}$  is overcome, with the result that cells swell and, in the case of erythrocytes, lyse. Such a mechanism operates in the case of complement-mediated lysis also and may do so in the case of cell-mediated lysis (Lachmann, 1983) as well. The fact that nonerythroid cells, which are capable of membrane repair, do not necessarily lyse when exposed to low amounts of virus, toxin or complement, but merely undergo the transient permeability changes described in the first part of this article, coupled with the fact that extracellular  $\text{Ca}^{2+}$  is able to inhibit pore-formation by these agents, opens up a new field of membrane research: a study of the effects of extracellular  $\text{Ca}^{2+}$ , as opposed to the currently much-studied effects of intracellular  $\text{Ca}^{2+}$ , on cellular function. Just as a study of viral genes paved the way to a better understanding of host genes, so a study of the toxin-like action of viruses at the cell surface may lead to a better understanding of plasma membrane function in healthy, as well as in diseased cells.

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## **An overview**

The last four decades have witnessed advances on many fronts in the area of amino acids, peptides and proteins. The depth of our understanding and the wealth of detail that has become available has certainly been fascinating. Yet, all this constitutes perhaps only what is a prelude because the scope of virgin territory that remains to be explored is vast and unending.

We should perhaps spare a few words in token appreciation of what has been accomplished. The text books still speak of twenty protein amino acids. And one may remember the classic review on amino acids by Vickery. Now, we recognize the existence of numerous amino acid derivatives as constituents of proteins. Such derivatization results from post-translational modification of proteins whether by enzymatic or non-enzymatic processes. The types of reactions that occur are many—alkylation, glucosylation, phosphorylation, carboxylation, hydroxylation and others. Thereby hangs many a tale concerning the intriguing functional role in the organism of such modification.

The past several years has seen investigators being confronted by an unending stream of what has been termed as the hit parade of peptides. And yet, looking back, when Bricas and Fromageot (1953) reviewed the area of naturally occurring peptides they were constrained to remark that the number then known was not very great and about the paucity of methods for isolating them in the pure state. They had also commented on certain physiologically active naturally occurring peptides being linked to the much larger protein molecules (“mother molecules”). Today we encounter a very large number of peptides which are the result of processing of proteins—such as zymogens, pro-proteins and pre-pro-proteins—by the cell. Certainly, there are others who owe their origin to other mechanisms in the cell. The peptides produced by lower organisms are also countless. We find many of them among the antibiotics.

Of methods for separation and isolation, and analysis there currently is no paucity. Automation has reduced the tedium. The ability to work on a micro scale has been enhanced. It is needless to enumerate specific techniques. Ion exchange and electrophoresis based methods and variations of chromatography such as HPLC and FPLC and affinity methods have been invaluable. Automated sequencing methods and micro manual sequencing methods, with exploitation of solid phase supports, have made structure determination of proteins relatively easy. With rapid sequencing methods for deoxyribonucleic acids becoming available some years ago, the fear had been expressed, that when the sequence of a protein could be so readily made out when the reading frame of a structural gene corresponding to the particular protein is known, regular sequencing methods for proteins would have become redundant. Well, developments in later years certainly bear out what an unwellfounded fear that was! Many hundreds of proteins have been sequenced and the data put to good use—*viz.* in working out evolutionary relationships. It is gratifying to note that large multienzyme

complexes such as the fatty acid synthetase are beginning to be tackled. Occasionally, we do encounter structures amongst small peptides which exhibit types of linkages which are unusual and not found in proteins. Difficulties in structure determination for the chemist with his chemical methods may be so great that only physical methods provide the solution, as in the case of thistrepton of molecular weight less than two thousand whose structure was solved (Hodgkin) by the X-ray method. Crystallographers have now solved with success details of architecture or the three dimensional structure of approximately 250 globular proteins. The classic contributions to this area by Ramachandran, Perutz, Kendrew, Phillips, Kartha and Harker, and Hodgkin—to name just a few—have had major impact in this field. When Biemann and Schemyakin were doing their pioneer studies on the use of mass spectrometry in elucidating peptide structure, few would have been hopeful about successful applications to large structures. Yet, the progress in recent years has been remarkable and one may find that both NMR and variations of mass spectrometry will have increasing roles to play in the future. Any way, the important structural themes in protein architecture seem to have been recognized. On the dynamics of folding and the dynamics of conformational states and transitions increasing attention is being devoted.

One of Anfinsen's contributions is the popular concept that primary structure of a protein determines its tertiary structure. Yet, one may still continue with the query how far is primary structure responsible for tertiary structure. Apart from some cytochromes which have disparate structure, and probably function, but share resemblances in tertiary structure there are instances such as tobacco mosaic virus protein, haemerythrin and apoferritin which are quite dissimilar but exhibit the same tertiary structure—a bundle of four nearly parallel  $\alpha$ -helices contributing a pattern not dictated by primary structure but by requirements of helix packing and spatial handedness (chiral connectivity). To account for this, it has been said that structures can be the result of convergence at the three dimensional level after divergence at the primary structure level.

Synthesis, if feasible or desirable, often follows on structure determination. The contribution of solution phase methods to peptide synthesis have been many. In both its manual and automated forms, solid phase synthesis as an innovation has had a very favourable impact. It is gratifying that Merrifield the proponent of solid phase methods has been honoured in 1984 with a Nobel Prize. As Kaiser puts it, "no longer is it necessary for a chemist interested in preparing a peptide 20 or 30 amino acids long to spend a substantial portion of his career in its construction . . . it is now possible for a graduate student in the course of his thesis studies to prepare as many as five to ten peptides of this size and to characterize their physical and biological properties". The technical advances in synthesis and separation make it now possible to test experimentally hypotheses on structure–function relationships, more thoroughly and with greater ease. Speaking of structure *versus* function, there is great and continuing activity in the study of enzymes, hormones, receptors, toxins and other macromolecules. A whole repertoire of chemical modification methods are brought to bear on the problem. Techniques range from the older unselective reagents, selective ones, affinity labels, photoaffinity labels, radiophotoaffinity labels, enzyme activated irreversible inhibitors (suicide substrates) to a variety of other probes. X-ray methods are exploited too.

On ribosomal and non-ribosomal pathways of protein and peptide synthesis there is great progress and continuing interest. That applies also to areas like tissue proteases and inhibitors, turnover, metabolism and associated metabolic disorders.

Against the above background of world-wide interest and activities in the area of peptides and proteins, the scientific community in India can view with some pride and concern the nature of the Indian effort and contributions. There is certainly much scope for greater participation and involvement in cultivating newer aspects of the field that at present have not many, a few, or no adherents. This particular issue of the *Journal of Biosciences* in no way represents the whole spectrum of activity in India, for obvious reasons. Topics that are covered in the seven papers that are included in the issue touch on binding of water and solutes to myosin, glucoamylase as examined by circular dichroism, the allosteric sites of mung bean aspartate transcarbamylase, bacterial and viral neuraminidases, the location of valinomycin in lipid vesicles, phosphofructokinase of rabbit liver and the nutritional quality of proteins. It is worth remarking that the September issue No. 3 of the journal contained five papers and the October issue No. 4 contained eight papers, out of a total of 12 and 15 papers respectively, dealing with proteins and peptides. It can be hoped that there will be ever increasing number of major contributions to the field in the future.

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## Thermodynamics of binding water and solute to myosin\*

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**Abstract.** From the isopiestic measurements of the extents of adsorption of water vapour by fish myosin at various values of water activities at three different temperatures, the changes in free energy, enthalpy and entropy of dehydration of the protein have been calculated. Extents of excess binding of solvent and solute to myosin have also been determined from isopiestic experiments in the presence of different inorganic salts, sucrose and urea respectively. Mols of water and solute respectively bound in absolute amounts to myosin have been evaluated from these data in limited range of solute concentrations. Free energy changes at different concentrations of these solutes have also been evaluated and their relations with 'salting-in' and 'salting-out' phenomena have been discussed. The order of the values of the standard free energy change for excess binding calculated with respect to an unified thermodynamic scale are found to be consistent with relative reactivity of binding water to myosin in the presence of inorganic salts, sucrose and urea.

**Keywords.** Hydration of myosin; thermodynamics of binding; Water-solute binding.

### Introduction

In the living system, muscle protein myosin in combination with actin remains in the form of biogel and is in constant interaction with water and various types of inorganic and organic solutes present in the aqueous phase. Change in mechanical energy during muscular expansion and contraction involves interaction of water and solute with this protein. The exact mechanism of this process is not yet clear. Myosin constitutes the major protein in animal and fish meat. During preservation of fish and meat with or without addition of salt, the role of water-protein interaction in preventing denaturation and maintaining the muscle texture and taste for long period becomes extremely important (Graham, 1977).

Myosin is insoluble in water but it becomes soluble at relatively high salt concentrations. Possibly for this reason, unlike other globular proteins, the hydration of myosin has not been studied in detail by the conventional physicochemical methods (Kuntz and Kauzmann, 1974). Compared to the globular shape of albumin and haemoglobin, the shape of myosin is highly elongated. The effect of this kind of change in shape on protein hydration needs critical examination.

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Abbreviation Used: BSA, bovine serum albumin.



Recently binding of water and various types of solutes to egg albumin (Bull and Breese, 1968, 1970a, b; Chatteraj and Mitra, 1977a, b), serum albumin (Mitra *et al.*, 1977),  $\beta$ -lactoglobulin and haemoglobin (Mitra *et al.*, 1978) respectively has been extensively studied using the isopiestic method. These studies of water vapour adsorption are found to be useful in evaluating many thermodynamic parameters related to the binding interactions of water to soluble as well as insoluble proteins. In the present study, thermodynamic aspects of binding water and solute to myosin have been studied on the basis of the data obtained from isopiestic experiments.

### Materials and methods

Myosin was isolated from the fresh water fish Rahu (*Labio rohita*) each weighing approximately 250–300 g. After descaling and beheading, the dorsal portion of the fish was isolated. It was then almost deboned, washed and macerated with mortar and pestle. The skin was left and 25 g of macerated muscle was washed three times with water. Following the standard method of isolation of cod myosin (Connell, 1962), the muscle was extracted with 250 ml of 0.10  $\mu$ M phosphate buffer at pH 6.4 for 30 min with slow stirring. The buffer also contained 0.47  $\mu$ M KCl, 0.10  $\mu$ M pyrophosphate and  $15 \times 10^{-3}$   $\mu$ M  $\text{MgCl}_2$ . The extract was then centrifuged at 3000 *g* for 15 min, the supernatant solution decanted and diluted 10-fold with water. The precipitated myosin gel was collected by centrifugation at 3000 *g* for 15 min. The precipitate was redissolved in the extractant with slow stirring and the solution diluted 10 times with water. The myosin gel was finally separated by centrifugation at 3000 *g* for 15 min. The extractant and water used in the above process were always ice cold. All the operations were done in cold.

After isolation, the protein was washed with cold distilled water to make it salt free and then lyophilised. Protein content of the lyophilised sample was found to be 100% (dry basis) as measured by the standard Kjeldahl method. The lyophilised sample was also examined for homogeneity by the usual procedure of gel electrophoresis using sodium dodecyl sulphate and polyacrylamide (Weber and Osborn, 1969). Along with the standard bands for myosin, the gel always indicated a faint band corresponding to the presence of actin. Thus, as in the case of cod myosin (Connell, 1962), in the present case also, the isolated myosin always contained a small fraction of actin as impurities. As in the case of cod myosin, the presence of the small fraction of actin has been ignored and it has been assumed that all the material precipitated by dilution is myosin. The lyophilised sample was stored in a desiccator at 4°C and directly used in the isopiestic experiments. All other chemicals were of analytical grade and these were used without further purification. Only urea was twice recrystallised from warm ethanol.

For the study of protein hydration in the absence of a solute, the isopiestic measurement was carried out as described in a previous publication (Chatteraj and Mitra, 1977a). Three to four days were sufficient for the attainment of isopiestic equilibrium. At the end of the experiment, mols of water ( $n_1$ ) adsorbed per kg of the dry protein sample was determined at isopiestic equilibrium from direct weighing. The concentration of sulphuric acid in the reference solution at equilibrium was estimated from which the relative humidity ( $p/p_0$ ) was known.

In the presence of inorganic salts, sugar and urea, the extent of protein hydration was measured using the same isopiestic technique as described in detail before (Mitra *et al.*, 1977). At isopiestic equilibrium, total mols of water ( $n'_1$ ) and total mols of solute ( $n'_2$ ) associated per kg of myosin (dry basis) were determined from direct weighing and the molality  $m_2$  of the solute in the reference solution was also estimated. It has been shown from thermodynamic considerations (Chattoraj and Mitra, 1977b) that  $m_2$  may also be treated as the molality of the free solute remaining dissolved in the free solvent in contact with myosin in the sample. This involves the assumption that the contribution of protein to vapour compared to that of the solute in the sample bottle is negligible. If  $X_1$  and  $X_2$  are mol fractions of the free solvent and solute respectively in the sample bottle then  $X_2/X_1$  becomes equal to  $m_2/55.51$ . We shall define mols of solvent and solute  $\Gamma_1^2$  and  $\Gamma_2^1$ , respectively, bound in excess per kg of myosin by the expression (Chattoraj and Mitra, 1977b; Mitra *et al.*, 1977)

$$\Gamma_1^2 = n'_1 - n'_2 \frac{X_1}{X_2} \quad (1)$$

and

$$\Gamma_2^1 = n'_2 - n'_1 \frac{X_2}{X_1} \quad (2)$$

Values of  $\Gamma_1^2$  and  $\Gamma_2^1$  can thus be estimated from the experimental data.

## Results and discussion

In figure 1, mols of water vapour ( $n_1$ ) adsorbed per kg of myosin at temperatures 45°C, 27°C and 10°C have been plotted against relative humidities  $p/p_0$ , ranging from zero to unity. These three isotherms are all sigmoid in nature. As in the case of other proteins, here also the sorption isotherms in figure 1 may be divided qualitatively into three classes. Value of  $n_1$  is approximately 2.5 mols of water per kg of myosin at 0.20 relative humidity and is independent of temperature. This amount of water is bound strongly to

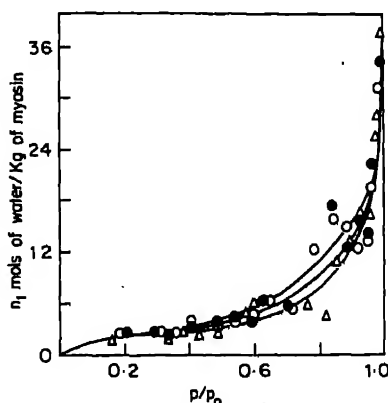


Figure 1. Plot of  $n_1$  vs.  $p/p_0$ . (O), 45°C; (●), 27°C; (Δ), 10°C.

the hydrophilic groups of protein forming a monolayer. According to Pauling (1945), each hydrophilic group is strongly bound to one molecule of water at this state of primary hydration. This strongly bound water behaves in many respects as part of the protein solid (Kuntz and Kauzmann, 1974). For relative humidity ranging between 0.20 and 0.70, water in excess of 2.5 mols per kg of protein are sorbed at new sites or holes and is responsible for the swelling of protein. The steep third part of the curve is probably due to the condensation of randomly oriented water molecules on the hydrated surface of proteins (Kuntz and Kauzmann, 1974).

The extrapolated value of  $n_1$  in figure 1 at  $p/p_o$  equal to unity is 40 mols of water per kg of myosin. This value of  $n_1$  (represented by  $\Delta n_1^o$ ) is independent of temperature. It may be regarded as the maximum and effective amount of water to be accommodated within the bound phase of protein-water systems thus completing various hydration shells (Chatteraj and Mitra, 1977a).

Average molecular weight, molecular length and molecular diameter of rod-shaped myosin are  $5 \times 10^5$  dalton,  $1.5 \times 10^{-7}$  and  $2 \times 10^{-9}$  meters respectively (Volkenshtein, 1983). From these free surface area  $A_f$  of myosin rods are calculated to be  $11.4 \times 10^{+5}$  square m/kg of the protein. Amount of water ( $\Delta n_1^o$ ) accommodated to the maximum extent per square meter of myosin is equal to  $\Delta n_1^o/A_f$  or  $3.5 \times 10^{-5}$  mol. Values of  $\Delta n_1^o$  and  $A_f$  for globular proteins bovine serum albumin (BSA), haemoglobin and egg albumin have been reported earlier (Chatteraj *et al.*, 1977a) so that  $\Delta n_1^o$  of these proteins are calculated to be  $2.9 \times 10^{-5}$ ,  $3.0 \times 10^{-5}$  and  $2.6 \times 10^{-5}$  mols per square meter respectively. Thus the water content per unit surface area of the globular and rod-shaped proteins are more or less of the same order and magnitude. If one assumes that a molecule of water is able to cover effectively  $10 \times 10^{-20}$  square meters of surface area then  $1.7 \times 10^{-5}$  mols of water will be needed to form a compact monolayer having one square meter surface area (Chatteraj, 1981). Thus it may appear that water molecules in compact form bound to the smooth protein surface at  $p/p_o$  close to unity tend to form a bilayer of  $H_2O$ . Most probably however, bound water molecules are not compact since the energies of interaction of water molecules with different types of hydrophilic and hydrophobic groups forming rough protein boundary are different.

According to the Raoult's law convention,  $p/p_o$  represents the activity ( $a_1$ ) of water in the system under consideration. As before, we will fix the standard state of reference (Chatteraj and Mitra, 1977a) for the system when  $a_1$  is unity and protein is surrounded by  $\Delta n_1^o$  mols of water and this in turn remain in equilibrium with free water present in the system. The integral free energy change ( $\Delta G_w$ ) due to the dehydration of protein molecules at activities lower than unity can then be calculated from the relation (Chatteraj and Mitra, 1977a; Chatteraj and Birdi, 1984)

$$-\Delta G_w = RT \int_{a_1=1}^{a_1} \frac{n_1}{a_1} da_1, \quad (3)$$

$-\Delta G_w$  at different values of  $a_1$  have been evaluated from the graphical integration using the experimental data. These are shown in figure 2 for various values of  $p/p_o$ . The integral free energy changes ( $\Delta G_w^o$ ) of dehydration for bringing water activities from unity to zero at different temperatures are presented in table 1.

From figure 2 as well as from table 1, one finds that the free energy change due to the

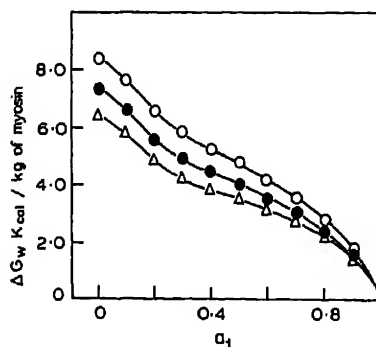


Figure 2. Plot of  $\Delta G_w$  vs.  $a_1$ . (O), 45°C; (●), 27°C; (Δ), 10°C.

Table 1. Standard free energy, enthalpy and entropy changes for dehydration of myosin.

Temperature °C	$\Delta G_w^\circ$ Kcal/kg myosin	$\Delta H_w^\circ$ Kcal/kg myosin	$T\Delta S_w^\circ$ Kcal/kg myosin	$\Delta S_w^\circ$ Cal/degree/kg myosin
10	6.4	-8.0	-14.4	-50
27	7.3	-9.0	-16.3	-54
45	8.3	-9.7	-18.0	-56

dehydration process is positive. Like all other proteins, dehydration of myosin is thus thermodynamically non-spontaneous process. At 27°C  $\Delta G_w^\circ$  for BSA, haemoglobin and egg albumin are +12.3, +9.4 and +9.6 Kcals per kg of protein (Chattoraj *et al.*, 1977a), respectively. Compared to this,  $\Delta G_w^\circ$  for rod-shaped myosin is +7.3 Kcals per kg of protein. Since surface area per kg of a protein in rod-shaped form is considerably higher than that in globular form, it seems that the surface free energy per unit area for myosin is considerably low. The structural stability of myosin, on the basis of the surface tensional forces discussed earlier (Chattoraj and Mitra, 1977a, b) is expected to be considerably lower than that of BSA. In agreement with this, the thermal denaturation temperature of myosin in the presence of 0.5 molar salt concentration is lower than 50°C (Chattoraj, D. K., Basu, S. and Das, K. P., unpublished results) whereas that for BSA in water is 65°C and it increases further with addition of neutral salt in the medium (Mitra and Chattoraj, 1978).

In the desorption process considered earlier (Chattoraj and Mitra, 1977a), one mol of hydrated protein in contact with unit mol fraction of free water is dehydrated reversibly until one mol of dry protein has been obtained.  $\Delta G_w^\circ$  is the standard free energy change for this desorption process whose value depends significantly on  $T$  but negligibly on  $p/p_0$  or  $n_1$  (Denbigh, 1971). The standard enthalpy change  $\Delta H_w^\circ$  for the dehydration may be calculated from the thermodynamic relation,

$$\frac{d(\Delta G_w^\circ/T)}{dT} = -\frac{\Delta H_w^\circ}{T^2}. \quad (4)$$

From the slope of the plot of  $\Delta G_w^\circ/T$  vs.  $T$ , values of  $\Delta H_w^\circ$  at different temperatures were calculated (vide table 1). Incorporating the values of  $\Delta H_w^\circ$  and  $\Delta G_w^\circ$  in the equation

$$\Delta G_w^\circ = \Delta H_w^\circ - T\Delta S_w^\circ \quad (5)$$

values of  $T\Delta S_w^\circ$  have been calculated (vide table 1). The adsorption and desorption of water on myosin seem to be significantly entropy controlled processes. Values of entropy change for desorption are included in table 1. It may be pointed out that the contribution of  $\Delta H_w^\circ$  in the dehydration process is also not negligible.

In figures 3 to 6, excess binding  $\Gamma_2^1$  for different solutes have been plotted as functions of  $X_2/X_1$ .  $\Gamma_2^1$  is negative for certain values of  $X_2/X_1$  but at several other values of the mol ratio composition,  $\Gamma_2^1$  becomes positive. It has been previously shown (Chatteraj

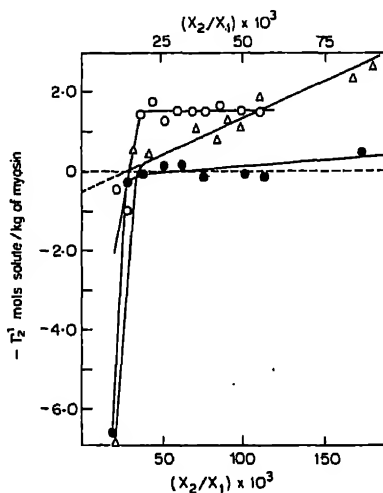


Figure 3. Plot of  $\Gamma_2^1$  vs.  $X_2/X_1$ . ( $\Delta$ ), KCl (left-upper scale); (O), NaCl (left-lower scale); ( $\bullet$ ), NaBr (left-lower scale).

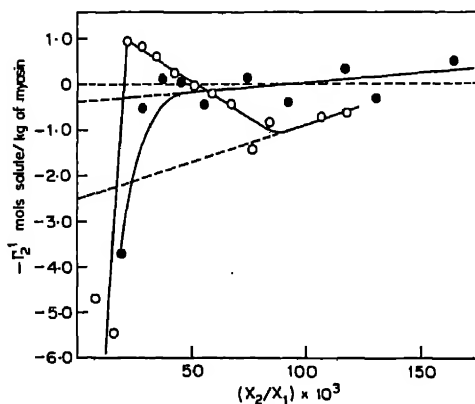


Figure 4. Plot of  $\Gamma_2^1$  vs.  $X_2/X_1$ . (O),  $\text{CaCl}_2$ ; ( $\bullet$ ), KI.

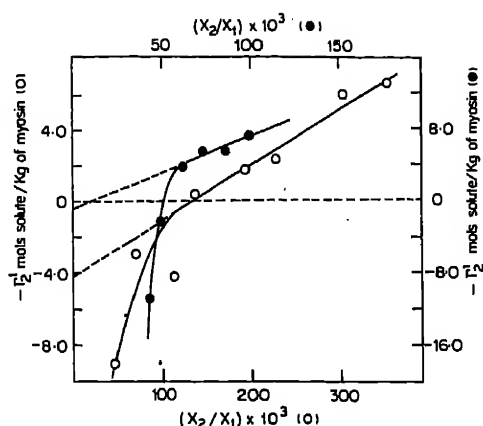


Figure 5. Plot of  $\Gamma_2^1$  vs.  $X_2/X_1$ . (O), urea; (●), KCNS.

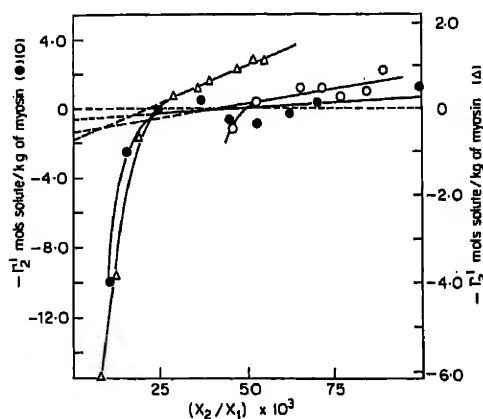


Figure 6. Plot of  $\Gamma_2^1$  vs.  $X_2/X_1$ . (O), LiCl; (●), sucrose; (Δ),  $\text{Na}_2\text{SO}_4$ .

and Mitra, 1977b; Chatteraj and Birdi, 1984) that

$$\Gamma_2^1 X_1 + \Gamma_1^2 X_2 = 0. \quad (6)$$

Thus, excess binding of water  $\Gamma_1^2$  becomes positive when  $\Gamma_2^1$  is negative and vice versa. We can further set  $n_1'$  and  $n_2'$  equal to  $n_1 + \Delta n_1$  and  $n_2 + \Delta n_2$  respectively (Chatteraj and Mitra, 1977b; Chatteraj and Birdi, 1984). Here  $\Delta n_1$  and  $\Delta n_2$  represent mols of solvent and solute respectively bound per kg of myosin whereas  $n_1$  and  $n_2$  are respective mols of the components per kg of protein remaining free. Inserting these in eqs (1) and (2), it can be shown that

$$\Gamma_1^2 = \Delta n_1 - \Delta n_2 \frac{X_1}{X_2} \quad (7)$$

and

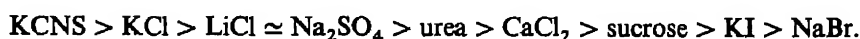
$$\Gamma_2^1 = \Delta n_2 - \Delta n_1 \frac{X_2}{X_1}. \quad (8)$$

As in the case of other proteins (Chatteraj and Mitra, 1977b; Mitra *et al.*, 1978; Sadhukhan and Chatteraj, 1983),  $\Gamma_2^1$  vs  $X_2/X_1$  plots for myosin in the presence of solutes are linear at certain ranges of composition (vide figures 3 to 6) so that  $\Delta n_1$  and  $\Delta n_2$  for these systems can be evaluated using eq. (8). These values of  $\Delta n_1$  and  $\Delta n_2$  in the presence of various solutes have been presented in table 2 along with standard errors. Values of  $\Delta n_2$  for  $\text{CaCl}_2$ ,  $\text{Na}_2\text{SO}_4$  and urea may have some significance but for other salts and sucrose, its value may be taken to be negligible in the light of the high error associated with  $\Delta n_2$ . Error in  $\Delta n_1$  for several solutes and particularly for LiCl, KI, KCNS and sucrose are also high. Such high errors also found in other systems (Bull and Breese, 1970b; Mitra *et al.*, 1978) are possibly associated with insolubility of proteins in the salt solution used.

**Table 2.**  $\Delta n_1$  and  $\Delta n_2$  for myosin in the presence of solutes.

Solute	$\Delta n_1$	$\Delta n_2$
	Mols water/kg myosin	Mols solute/kg myosin
LiCl	$34 \pm 17$	$1.4 \pm 1.2$
KCl	$36 \pm 7$	$0.5 \pm 0.4$
KCNS	$80 \pm 18$	$0.8 \pm 1.4$
KI	$4 \pm 2$	$0.4 \pm 0.2$
NaBr	$3 \pm 1$	$0.2 \pm 0.1$
$\text{CaCl}_2$	$16 \pm 7$	$2.5 \pm 0.7$
Urea	$31 \pm 3$	$4.2 \pm 0.7$
Sucrose	$13 \pm 9$	$0.7 \pm 0.5$
$\text{Na}_2\text{SO}_4$	$34 \pm 2$	$0.7 \pm 0.1$

Electrolytes and organic solutes stand in the following order in terms of magnitudes of  $\Delta n_1$



This order is not in agreement with that expected from the lyotropic series. Lyotropic series in terms of  $\Delta n_1$  remains qualitatively valid for BSA and egg albumin with reference to many inorganic salts (Bull and Breese, 1970a; Mitra *et al.*, 1977). Except KCNS, values of  $\Delta n_1$  for myosin with different solutes are always less than  $\Delta n_1^0$ . Thus solutes dehydrate myosin in general and the extent of dehydration is dependent on the nature of the solute.

Deviation of the plots from linearity shown in figures 3 to 6 in certain regions of  $X_2/X_1$  is an indication that  $\Delta n_1$  or  $\Delta n_2$  or both vary now with change of  $X_2/X_1$  (Chatteraj and Mitra, 1977b; Chatteraj and Birdi, 1984). For a given solute, values of  $\Gamma_2^1$  in eq. (8) may become positive when  $\Delta n_2/\Delta n_1$  is greater than  $X_2/X_1$  in certain ranges of concentrations whereas in other concentration ranges,  $\Gamma_2^1$  may be negative when  $\Delta n_2/\Delta n_1$  is less than  $X_2/X_1$ . Further, for each solute, there exists a definite value of  $X_2/X_1$  (or  $m_2$ ) when  $\Gamma_2^1$  becomes zero (vide figures 3 to 6). This is the azeotropic state (Chatteraj and Birdi, 1984) for binding of solvent and solute to myosin when  $\Delta n_2/\Delta n_1$  becomes equal to  $X_2/X_1$ . Values of  $m_2$  of various solutes at the azeotropic states

presented in table 3 are different from each other. It may be of interest to note that for  $\text{CaCl}_2$  there exist two azeotropic points. The significance and detailed analysis of the azeotropic states in terms of arrangement of water and solute molecules in the bound phase of a protein need detailed analysis in the future.

**Table 3.** Azeotropic points and standard free energies of excess hydration of myosin in presence of solute.

Solute	$m_2$ at azeotropic point	$\Delta G_{ws}^\circ$ Kcal/kg myosin	$\Gamma_1^\pi$ Mols water/kg myosin
KCNS	2.83	22	70
NaCl	1.72	14	
$\text{CaCl}_2$	1.11	11	43
	2.83		
KCl	0.99	10	25
$\text{Na}_2\text{SO}_4$	1.36	5.6	20
LiCl	2.77	4.4	17
Urea	7.54	4.2	15
Sucrose	2.77	-3.4	3
NaBr	3.33		1.5
KI	5.55		1.4

The free energy change  $\Delta G_{ws}$  in bringing myosin from the surrounding of water to that of a solution of composition  $X_1$  (or  $X_2$ ) has been calculated from the thermodynamic relation (Chattoraj and Mitra, 1979)

$$\Delta G_{ws} = RT \int_{f_1 X_1 = 1}^{f_1 X_1} \frac{\Gamma_1^2}{f_1 X_1} d(f_1 X_1) + RT \Gamma_1^2 \ln \frac{f_1 X_1}{0.5}. \quad (9)$$

Following the special method given earlier by Chattoraj and Mitra (1979), the integral in eq. (9) has been solved graphically between appropriate limits using values of  $\Gamma_2^1$  and  $X_2$ .  $\Delta G_{ws}$  is the free energy change when  $\Gamma_2^1$  mols of solute (or  $\Gamma_1^2$  mols of solvent) are transferred in excess to the bound phase due to the change of mol fraction of the solvent from unity to 0.5 (or solute mol fraction from 0.0.5).

In figures 7 and 8, values of  $\Delta G_{ws}$  thus calculated from the experimental data have been plotted (for some solutes) against  $X_2/X_1$ .  $\Delta G_{ws}$  is zero when myosin is in contact with pure water. With addition of a solute,  $\Delta G_{ws}$  becomes at first negative. From the treatment of the Gibbs adsorption equation (Chattoraj and Mitra, 1977b, 1979), this means that the attractive interaction between the protein molecules themselves is reduced and protein-solvent interaction is increased with addition of relatively small amount of solute to the medium. This is in accordance with the salting-in phenomena for protein dissolution (Chattoraj and Mitra, 1979). Myosin is insoluble in pure water but begins to dissolve when the ionic strength of the solution is gradually increased. With increase of solute concentration or  $X_2/X_1$  further,  $\Delta G_{ws}$  gradually becomes less



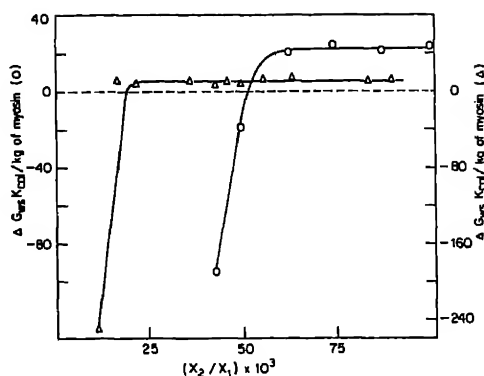


Figure 7. Plot of  $\Delta G_{ws}$  vs.  $X_2/X_1$ . (O), KCNS; ( $\Delta$ ), KCl.

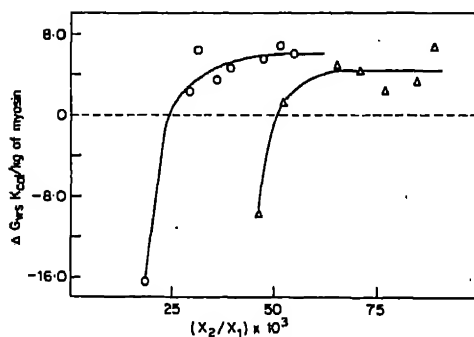


Figure 8. Plot of  $\Delta G_{ws}$  vs.  $X_2/X_1$ . (O),  $\text{Na}_2\text{SO}_4$ ; ( $\Delta$ ), LiCl.

negative, zero and finally it becomes positive (vide figures 7 and 8). In terms of the concept of the Gibbs adsorption equation, this means that with increase of solute concentration to a large extent, protein-solvent interaction decreases considerably so that there is a tendency for the protein molecules to aggregate and precipitate out by attractive interaction. This feature is consistent with the salting out phenomena observed for many proteins (Chattoraj and Mitra, 1979).

At considerably higher values of  $X_2/X_1$ ,  $\Delta G_{ws}$  in the presence of each solute approaches a limiting constant value  $\Delta G_{ws}^0$ . At this state,  $\Gamma_1^2$  also reach steady constant values  $\Gamma_1^m$  for different solutes.  $\Gamma_1^m$  values for different solutes are presented in table 3.  $\Delta G_{ws}^0$  may be regarded as the standard free energy change for protein hydration at the state of saturation of the bound phase (Chattoraj and Birdi, 1984; Chattoraj and Mitra, 1979) when mol fraction of the solvent changes from unity to 0.5.  $\Delta G_{ws}^0$  values thus presented in table 3 are comparable with each other since the thermodynamic scale is a perfectly general one. The order of solutes in terms of  $\Delta G_{ws}^0$  is



This order is consistent with the order of solutes in terms of  $\Gamma_1^m$  representing maximum extent of binding interaction of the proteins with solute and water.

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## Structure and stability of glucoamylase II from *Aspergillus niger*: A circular dichroism study

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**Abstract.** Glucoamylase II (EC 3.2.1.3) from *Aspergillus niger* has 31%  $\alpha$ -helix, 36%  $\beta$ -structure and rest aperiodic structure at pH 4.8 as analysed by the method of Provencher and Glockner (1981, *Biochemistry*, 20, 33). In the near ultra-violet circular dichroism spectrum the enzyme exhibits peaks at 304, 289, 282 and 257 nm and troughs at 285, 277 and 265 nm respectively. The enzyme activity and structure showed greater stability at pH 4.8 than at pH 7.0, were highly sensitive to alkaline pH but less sensitive to acid pH values. The enzyme retained most of its catalytic activity and structure even on partial removal of carbohydrate moieties by periodate treatment but was less stable at higher temperatures and storage at 30°C. Reduction of the periodate treated enzyme did not reverse the loss of stability. Binding of the synthetic substrate, *p*-nitrophenyl- $\alpha$ -D-glucoside, perturbed the environment around aromatic amino acids and caused a decrease in the ordered structure.

**Keywords.** *Aspergillus niger*; glucoamylase; circular dichroism; carbohydrate moieties.

### Introduction

Several glucoamylases ( $\alpha$ -1,4 glucan glucohydrolase, EC 3.2.1.3) were purified to homogeneity from different fungal sources by us and some of their biochemical and biophysical characteristics were reported (Manjunath and Raghavendra Rao, 1979). Among these enzymes, glucoamylase II, which is the major component from *Aspergillus niger* is a glycoprotein with 18% carbohydrate and with pH and temperature optima of 4.8 and 60°C, respectively. Very little information is available on the structure-function relationships in these glucoamylases. As a part of our ongoing programme of investigations on glucoamylases, we report in this communication the results of our studies on the conformation and stability of the enzyme using near and far ultra-violet (UV) circular dichroism (CD) and activity measurements of the enzyme as a function of pH, temperature and substrate concentration. The effects of a partial removal of the carbohydrate component by periodate oxidation and subsequent reduction either with sodium borohydride or with hydrogen in the presence of platinum oxide on the physical and catalytic properties are also presented here.

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Abbreviations used: UV, Ultra-violet; CD, circular dichroism; BSA, bovine serum albumin; hCG, human chorionic gonadotropin.

## Materials and methods

Glucozyme a preparation of glucoamylase from *A. niger* was a gift from Anil Starch Products, Ahmedabad. Sodium metaperiodate, *p*-nitrophenyl- $\alpha$ -D-glucoside and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, Missouri, USA. Guanidine HCl was from Pierce Chemical Co., Rockford, Illinois, USA. All other reagents and chemicals used were of analytical grade.

Glucoamylase II from *A. niger* was purified to homogeneity from glucozyme by the method of Manjunath and Raghavendra Rao (1979). Enzyme activity was determined using a 2% (w/v) starch solution. One unit of enzyme activity is defined as the amount of enzyme required for the liberation of 1  $\mu$ mol of glucose per min at 60°C. Enzyme activity was also determined using the synthetic substrate *p*-nitrophenyl- $\alpha$ -D-glucoside (Sternberg, 1970).

Protein was determined both by the method of Lowry *et al.* (1951) using BSA as the standard and also from the absorbance of the solution at 280 nm using a value of  $A_{1\%}^{1\text{cm}} = 13.8$ . This value was obtained by measuring the absorbance of a series of solutions whose concentrations were determined by the microKjeldahl procedure for the estimation of nitrogen. A value of 6.25 was used for converting nitrogen to protein. Total carbohydrate was estimated by the phenol-sulphuric acid reaction (Dubois *et al.*, 1956). Half-cystine content was determined as cysteic acid after performic acid oxidation according to Moore (1963). Total free -SH groups were estimated by the method of Ellman (1959). The total amino groups was estimated by the method of Habeeb (1966) using 2,4,6-trinitrobenzenesulphonate. Tryptophan content of the enzyme was determined spectrophotometrically according to Edelhoch (1967) in 6 M guanidine HCl. The total aldehyde moieties which were produced by the oxidation of the enzyme with periodate were estimated by the cyanometric method of Dyer (1956).

Periodate oxidation was carried out according to Yasuda *et al.* (1971). Appropriate samples of the enzyme (4 mg/ml) were incubated at room temperature in the dark with 0.1 M sodium metaperiodate and in 0.05 M acetate buffer, pH 4.8. After 30 min the reaction was stopped by the addition of a 5 molar excess of thioglycolate. The enzyme was then recovered after extensive dialysis at 4°C against 0.05 M acetate buffer, pH 4.8.

Two methods of reduction of the periodate treated enzyme were tested. The enzyme was reduced with  $\text{NaBH}_4$  by a modification of the procedure described by Carlson (1966). Freeze-dried enzyme (1 mg/ml) in 0.1 M phosphate buffer, pH 7.0 was treated with 0.3 M  $\text{NaBH}_4$  at 37°C and the pH was maintained at 7.0 using 1 M HCl. After 50 h, the excess of borohydride was destroyed in the cold by the addition of glacial acetic acid to pH 5.0 and the solution was dialysed overnight against 0.05 M acetate buffer pH 4.8 at 4°C with four changes.

The enzyme was reduced with platinum oxide and hydrogen according to Huennekens *et al.* (1963). Platinum oxide (20 mg) was suspended in 2.5 ml of 0.05 M acetate buffer, pH 4.8 in a hydrogenation flask. The catalyst was reduced at 25°C and at atmospheric pressure until no more hydrogen was absorbed. The enzyme (10 mg) suspended in 2.5 ml of acetate buffer, pH 4.8 was introduced into the hydrogenation flask. After 4 to 5 h of hydrogenation, the apparatus was flushed with nitrogen. The reduced enzyme was then filtered to remove the catalyst.

### CD measurements

CD measurements were made in a JASCO-J-20C automatic recording spectropolarimeter calibrated with d-10-camphor sulphonic acid. Quartz cells of different path length (1 cm, 0.1 cm or 0.05 cm) were used for measurements in the region 350 to 200 nm. Slits were programmed to yield a band width of 10 Å at each wavelength. Mean residue ellipticities  $[\theta]_{\text{mrw}}$  were calculated by standard procedures (Adler *et al.*, 1973). A value of 110 for mean residue weight was used. The CD spectra were analysed by the method of Provencher and Glockner (1981) to estimate the secondary structure.

For making measurements at different temperatures, the solution was heated to the desired temperature by circulating water through a double walled cell holder from a preheated water-bath and then allowed to remain at that temperature for 15 to 20 min for attainment of thermal equilibrium before making measurements. Concentrations of protein solutions were adjusted to give 0.2–1.5 absorbance at 280 nm in 10 mm light path quartz cells. All the measurements were made in triplicate and at room temperature unless mentioned otherwise.

## Results and Discussion

### Conformation and storage stability of glucoamylase

**Effect of pH:** The CD spectrum of the enzyme in 0.05 M acetate buffer at pH 4.8, which is the optimum pH for the activity of the enzyme, is shown in figure 1. In the near UV region the enzyme exhibited peaks at 304, 289, 282, 265 and 257 nm and troughs at 285, 277 and 268 nm (figure 1A). Chemical and spectrophotometric assays showed that the enzyme contained 13 tryptophan, 14 tyrosine, 19 phenylalanine and 8 half-cystine residues per mol of the enzyme (Shenoy, B.C., unpublished results; Manjunath and Raghvendra Rao, 1979). The bands at 289, 285, 282 and 277 are probably due to tryptophan and tyrosine. Individual assignment of the bands is difficult because of the overlapping nature of tyrosine and tryptophan absorption bands. The bands at 259, 265 and 267 nm are due to phenylalanine and that at 304 nm may also possibly be due to tryptophan. The overlapping contribution of cystine bands in this region cannot be ignored (Strickland, 1974). The fine spectral features of the enzyme was retained even after storing the enzyme at 4°C for 10 days at pH 4.8 (figure 1A) indicating little or no conformational changes around the aromatic amino acid residues in the enzyme. The enzyme activity remained unaltered (table 1).

The far UV CD spectrum of the enzyme at pH 4.8 in 0.05 M acetate buffer is shown in figure 1B. The enzyme has minima at 218 nm and at 210 nm. Analysis of the secondary structure of the enzyme by the method of Provencher and Glockner (1981) indicated that the enzyme contained 31 %  $\alpha$ -helix, 36 %  $\beta$ -structure and 33 % remainder (table 1). The figure 1C shows the computer fit data of the enzyme by the Provencher and Glockner (1981) method. By the curve-fitting procedure of Greenfield and Fasman (1969) it was possible to fit the experimental data fairly well with the assumed value of 15 %  $\alpha$ -helix, 40 %  $\beta$ -structure and rest aperiodic. Compared to the value obtained by the method of Provencher and Glockner (1981), helical content appears considerably low. For further comparison, only the method of Provencher and Glockner (1981) was

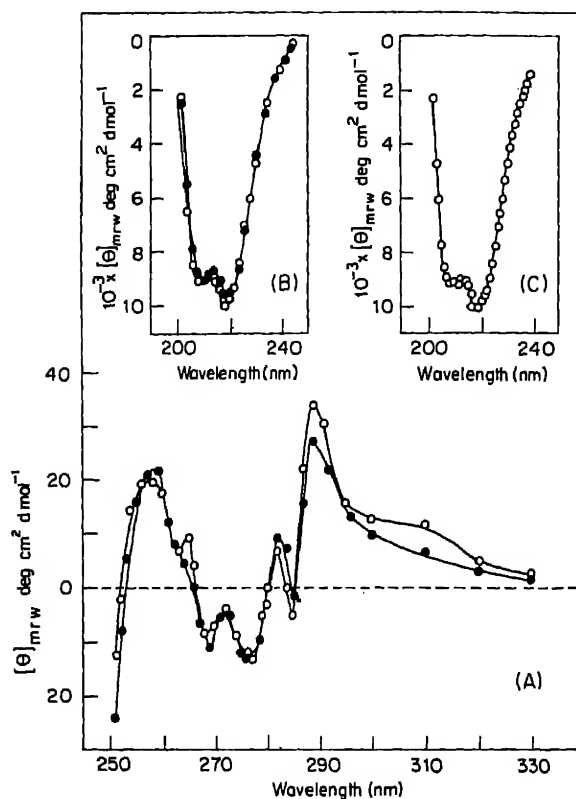


Figure 1. A. Near UV CD spectrum of glucoamylase II at pH 4.8. (O), Native (fresh); (●), native after 10 days. B. Far UV CD spectrum of glucoamylase II at pH 4.8. (O), Native (fresh); (●), native after 10 days. C. Far UV CD spectrum of glucoamylase II. (O), Experimental; (—), calculated (by curve fitting procedure of Provencher and Glockner, 1981).

used in view of the fact that the estimation of the helix and  $\beta$ -sheet by the method of Provencher and Glockner is more accurate than by other methods. After storing the native enzyme at 4°C for 10 days at pH 4.8, there was a decrease in the helical content of the enzyme from 31%  $\alpha$ -helix to 22%  $\alpha$ -helix and an increase in the  $\beta$ -structure from 36% to 55% without any loss of activity (figure 1B).

The near UV CD spectrum of the enzyme at pH 7.0 in 0.016 M phosphate buffer is shown in figure 2A. Compared with the spectrum of the enzyme at pH 4.8, the 282 nm band is absent, the intensity of the 290 nm band was much less and the intensities of the bands at 285, 277 and 269–70 nm increased with the appearance of a new band at 274 nm. The near UV CD spectrum indicated a change in the environment of aromatic amino acids due to an increase in the pH from 4.8 to 7.0. At pH 7.0, the enzyme has only 2% of its activity at pH 4.8. But when the pH is readjusted to 4.8, the enzyme regained all the activity indicating that the changes in conformation was probably reversible. On storing the enzyme for 10 days at pH 7.0, no significant changes were noticed in the near UV CD spectrum and the activity of the enzyme (assayed at pH 4.8) decreased to about

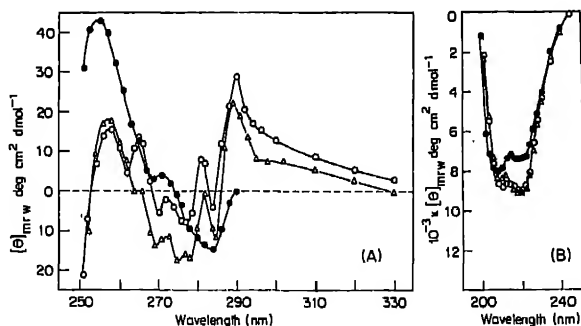
**Table 1.** Effect of various physical and chemical treatments on conformation and activity.

	Helical content*			Activity (%)
	$\alpha$	$\beta$	Random	
<i>Native enzyme (pH 4.8, 60°C)</i>				
Native (fresh)	31	36	33	100
After 10 days	22	55	23	100
<i>pH (60°C)</i>				
pH 2.0	22	60	18	61
pH 7.0 (fresh)	24	52	24	2
pH 7.0 (after 10 days)	41	34	25	2
pH 10.0	17	38	45	0
<i>Temperature (pH 4.8)</i>				
15.5°C	20	43	37	0
30.5°C	28	36	36	22
60°C	25	34	41	100
<i>Heat treatment (pH 4.8, 60°C)</i>				
Heat treated at pH 4.8 (60°C, 20 min)	20	47	33	60
<i>Periodate treatment (pH 4.8, 60°C)</i>				
Periodate treated (fresh)	27	24	49	60
Periodate treated (after 10 days)	17	27	56	0
Periodate + heat treated at pH 4.8 (60°C, 20 min)	3	49	48	0
<i>Substrate (pH 4.8, 60°C)</i>				
Enzyme + <i>p</i> -nitrophenyl- $\alpha$ -D-glucoside	27	41	32	100
	26	38	36	100

Activities of the enzyme were determined at pHs and temperatures mentioned. Substrate in all cases except 6 was soluble starch. Details in text.

\* By the method of Provencher and Glockner (1981).

87% of the original. The far UV spectrum of the enzyme (figure 2B) suggests an increase in the helical content of the enzyme on storage at pH 7.0 for a period of 10 days (table 1). Although there was an increase in the helical content of the enzyme upon storage (at pH 7.0), activity decreased as mentioned above.



**Figure 2.** A. Near UV CD spectrum of glucoamylase II at various pH values. (O), pH 2.0; ( $\Delta$ ), pH 7.0; ( $\bullet$ ), pH 10.0. B. Far UV CD spectrum of glucoamylase II at various pH values. (O), pH 2.0; ( $\Delta$ ), pH 7.0; ( $\bullet$ ), pH 10.0.



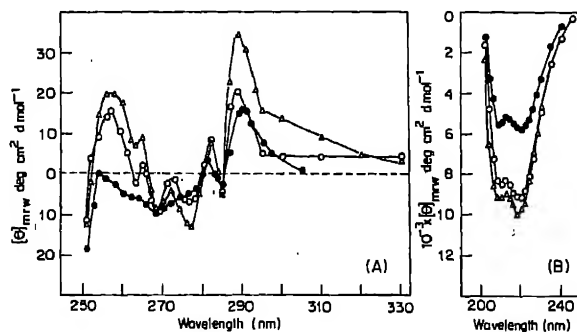
When the pH of the enzyme solution was raised to 10.0, changes in near UV CD bands (figure 2A) were observed which reflects changes in the conformation of the enzyme. The intensities and position of the band at 290, 282, 276 and 258 nm change remarkably indicating the possible ionisation of the tyrosine residues or changes in the environment around aromatic amino acids. The far UV CD spectrum (figure 2B) suggested that there was a progressive loss of secondary structure upon increasing the pH (table 1). The enzyme activity was completely and irreversibly lost at pH 10.0. At pH 2.0, the enzyme still retained 60% of its activity (table 1). The CD spectrum in both far UV and near UV CD (figure 2) suggested that the enzyme was fairly stable at acid pH.

#### *Effect of temperature*

In the near UV CD spectrum of the enzyme in the temperature range 15.5°C–60°C, the only change at pH 4.8 was the disappearance of 285 nm band at 15.5°C. The far UV CD spectrum showed that there was a decrease in helical content of the enzyme at 15.5°C and also a slight decrease at 60°C (table 1). This remarkable stability towards heat may possibly be due to a combination of low pH (4.8) and carbohydrate moiety. The other factors which impart thermal stability to protein molecules, such as hydrophobicity (Mozhev and Martinek, 1984) and abundance of disulphide bonds as in RNase are not likely to be significant in this instance. As this enzyme has very few disulphide linkages and secondly even though hydrophobicity increases on removal of the carbohydrate moieties, the stability of the enzyme towards heat and storage is reduced significantly.

#### *Effect of partial removal of carbohydrate moieties*

The native enzyme contains 18% total carbohydrates as estimated by Dubois *et al.* (1956) method. After periodate treatment, the carbohydrate content of the enzyme was reduced to 4.5% (table 1). The enzyme after partial removal of carbohydrate retained most of the near UV CD bands (figure 3A) intact with only a slight decrease in the intensity of the bands indicating that removal of carbohydrate moieties did not significantly change the conformation around aromatic amino acids. The periodate



**Figure 3.** A. Near UV CD spectrum of glucoamylase II. (Δ), Control; (○), periodate treated (fresh); (●), periodate treated after 10 days. B. Far UV CD spectrum of glucoamylase II. (Δ), Control; (○), periodate treated (fresh); (●), periodate treated after 10 days.

treated enzyme retained 60% of the original activity. However, the storage stability of such an enzyme preparation to storage was poor since complete loss of activity occurred at 4°C in 10 days. In the near UV region, 276, 265 and 258 nm bands were absent in the spectrum of the periodate treated enzyme. Also the intensity of 282 and 289 nm bands were decreased. These results indicate that the environment around the aromatic amino acids changes considerably after storage of the deglycosylated enzyme.

Immediately after removal of the carbohydrate moiety there was no significant change in the secondary structure of the enzyme at pH 4.8 (figure 3B). But after storing at pH 4.8 for 10 days at 4°C, the enzyme after partial removal of carbohydrate lost considerable amount of the secondary structure which was also reflected in the near UV CD spectrum of the enzyme. The  $\alpha$ -helix content decreased from 31% to 17%. Activity loss at this stage was almost complete. Thus it appears that the carbohydrate moiety plays an important role in stabilizing the enzyme conformation. It should be mentioned here that periodate treatment did not lead to any change in the amino acid content of the enzyme. The contents of tryptophan, tyrosine, methionine and half-cystine residues remained the same (Shenoy, B. C. unpublished results).

There was no change in the number of amino groups (8 in the untreated enzyme) immediately after treatment of the enzyme with periodate when 48 aldehyde groups were formed. But on storage for 10 days the amino groups were reduced to 4 from 8 along with the reduction in the aldehyde groups from 48 to 44. No free-SH groups were detected. Gross changes in secondary structure were brought about perhaps by the cross-linking of  $\epsilon$ -amino groups of lysine with the aldehyde groups resulting in a total loss of activity (table 2).

**Table 2.** Effect of periodate oxidation and reduction in presence of hydrogen and platinum oxide (as catalyst).

	Helical content			CH <sub>2</sub> O (%)	Aldehyde groups mols/mol	Amino groups mols/ mol	SH groups mols/ mol	Activity (%)
	$\alpha$	$\beta$	Random					
<i>Native enzyme</i>								
Native (fresh)	31	36	33	18	Nil	8	Nil	100
After 10 days	22	55	23	18	Nil	8	Nil	100
<i>Periodate treatment</i>								
Periodate treated (fresh)	27	24	49	4.5	48	8	Nil	60
After 10 days	17	27	56	4.5	44	4	Nil	0
<i>Reduced in presence of hydrogen and platinum oxide</i>								
Fresh	6	44	50	18	Nil	8	Nil	142
After 10 days	6	44	50	18	Nil	8	Nil	142
<i>Periodate + reduced in presence of hydrogen and platinum oxide</i>								
Fresh	5	45	50	4.5	Nil	8	Nil	75
After 10 days	5	45	50	4.5	Nil	8	Nil	70

The effect of heating the native enzyme and the enzyme after partial removal of carbohydrate at 60°C for 20 min (removal of carbohydrate moieties by periodate treatment and the effect of heat on the periodate treated enzyme) on the CD spectrum in both near UV and far UV region in 0.05 M acetate buffer, pH 4.8 are shown in figure 4A and 4B. From figure 4, it is clear that heating (or partial removal of carbohydrate) leads to decrease in the helical content of the enzyme in far UV CD at pH 4.8. But there was a considerable (about 40%) decrease in the activity of the enzyme (table 1) which was reflected in the far UV CD band and in near UV CD band intensities. The change in intensity of the band at 276 nm and 257 nm indicated some change in conformation of the enzyme. The combined effect of periodate and heat treatment, caused gross conformational changes in the enzyme and leading to a complete loss of activity (table 1). Many enzymes are known to be glycoproteins (Spiro, 1973). Glucoamylases of fungi, studied so far, seem to belong to this category (Manjunath and Raghavendra Rao, 1979; Pazur *et al.*, 1963). Carbohydrate moieties in glycoproteins are known to confer generally resistance to proteolysis (Hayashida, 1975; Hayashida and Yoshino, 1978; Yoshino and Hayashida, 1978), loss of activity upon storage and confer structural stability in many instances against heat and pH, *viz.*, glucoamylase of *A. niger* (Manjunath *et al.*, 1983; Pazur *et al.*, 1970) invertase of *Neurospora crassa* (Gascon *et al.*, 1968) cellulases of a thermophilic fungus (Hayashida and Yoshioka, 1980). In contrast to these observations, removal of carbohydrate moieties resulted in an increase in the thermal stability of human chorionic gonadotropin (hCG) perhaps by increasing the hydrophobicity of hCG (Manjunath and Sairam, 1983). Therefore, it is reasonable to conclude from the results presented that the carbohydrate moieties apparently confer conformational/structural stability towards heat and pH in the present instance.

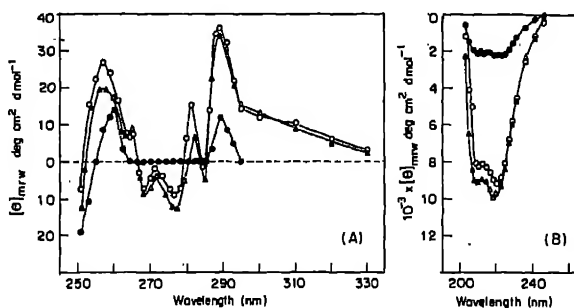


Figure 4. A. Near UV CD spectrum of glucoamylase II, ( $\Delta$ ), Control; (O), heat treated; ( $\bullet$ ), periodate and heat treated. B. Far UV CD spectrum of glucoamylase II. ( $\Delta$ ), Control; (O), heat treated; ( $\bullet$ ), periodate and heat treated.

### Effect of reduction

It is probable that the aldehyde moieties produced by periodate oxidation react during storage with the free amino groups ( $\epsilon$ -amino groups of lysine) in the polypeptide leading to loss of conformation and activity. In order to ascertain whether reduction of the aldehyde moieties to primary alcohol groups would improve the stability of the enzyme to heat/storage, the oxidized enzyme was reduced with sodium borohydride at

pH 7.0 for 50 h. But on reduction with sodium borohydride, the enzyme lost its activity completely. Hence a milder reducing agent, hydrogen in the presence of platinum oxide was used to reduce the oxidized enzyme. This enzyme was stable for a period of 10 days but lost its activity completely after a month. No free -SH groups were found in oxidized enzyme after reduction. Activity of the reduced control as well as reduced periodate-treated enzyme was higher than that of the respective controls (table 2). At this stage, it is not possible to explain the cause of this increase in activity.

On reduction of the control enzyme with hydrogen in the presence of platinum oxide as catalyst, there are changes in the near UV CD bands (figure 5A) which reflect changes in the conformation of the enzyme. The band intensities at 258, 290 nm changed remarkably with the disappearance of 265 and 282 nm bands. Activity of the enzyme increased to 142% of the control. Gross changes in helical structure are seen (figure 5B). On reduction of the periodate treated enzyme the band intensities at 258, 269 and 289 nm change were markedly accompanied by a disappearance of 282 nm band. Helical structure was also destroyed completely (figure 5B). No free -SH groups could be detected. Enzyme activity was lost completely after a month, but not in 10 days. The reduction of the oxidized enzyme only helped to prolong the storage stability, but like the oxidized enzyme, it is unstable at higher temperatures. It appears that carbohydrate groups may be necessary for maintaining native conformation of the enzyme.

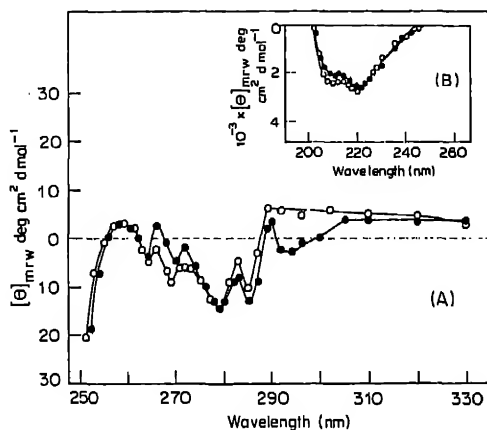
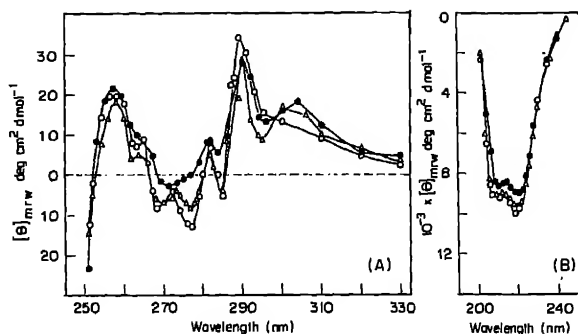


Figure 5. A. Near UV CD spectrum of glucoamylase II. (O), Native enzyme reduced; (●), periodate treated and reduced. B. Far UV CD spectrum of glucoamylase II. (O), Native enzyme reduced; (●), periodate treated and reduced.

### Effect of substrate

The effect of a synthetic substrate *p*-nitrophenyl- $\alpha$ -D-glucoside on the CD spectrum of the enzyme is shown in figure 6. Although the effects at different concentrations of substrate were measured, only the data at the highest concentration (0.3 mM) is given. The lowest concentration which could be used was 0.1 M, the optical activity of the material determining this limit. *p*-Nitrophenyl- $\alpha$ -D-glucoside has CD bands in both



**Figure 6.** A. Near UV CD spectrum of glucoamylase II in the presence of substrate. (O), Control; (Δ), enzyme + substrate ( $1 \cdot 10^{-4}$  M concentration); (●), enzyme + substrate ( $3 \cdot 10^{-4}$  M concentration). B. Far UV CD spectrum of glucoamylase II in the presence of substrate. (O), Control; (Δ), enzyme + substrate ( $1 \cdot 10^{-4}$  M concentration); (●), enzyme + substrate ( $3 \cdot 10^{-4}$  M concentration).

near UV (300 nm) and far UV (220 nm), but the reaction products D-glucose and *p*-nitrophenol have none in these regions. Appropriate corrections were made for the optical activity of the substrate. From the figure 6A it is clear that, particularly in the presence of the substrate, the bands at 276 and 285 nm are affected considerably. It appears that substrate binding probably occurs at tryptophan and/or tyrosine residues which alters the environment around the tryptophan/tyrosine residues.

Earlier Freedberg *et al.* (1975) reported the isolation and characterisation of an exo-1,4-glucosidase from *A. niger*, with a molecular weight of 62,000. CD measurements of the enzyme revealed 15–25%  $\alpha$ -helix in addition to  $\beta$ -structure and random coil. When the enzyme reacts with either synthetic substrate or inhibitor, there is a small but significant decrease in secondary structure (Freedberg *et al.* 1975). Our results are in conformity with those of Freedberg *et al.* (1975).

It could be concluded that the 18% carbohydrate content of the glucoamylase from *A. niger* may have an important function in stabilizing the conformation/structure of the enzyme as well in protecting the enzyme against loss of activity upon storage or due to changes in the solvent environment.

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## Interaction of rose bengal with mung bean aspartate transcarbamylase

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**Abstract.** The fluorescein dye, rose bengal in the dark: (i) inhibited the activity of mung bean aspartate transcarbamylase (EC 2.1.3.2) in a non-competitive manner, when aspartate was the varied substrate; (ii) induced a lag in the time course of reaction and this hysteresis was abolished upon preincubation with carbamyl phosphate; and (iii) converted the multiple bands observed on polyacrylamide gel electrophoresis of enzyme into a single band. The binding of the dye to the enzyme induced a red shift in the visible spectrum of dye suggesting that it was probably interacting at a hydrophobic region in the enzyme. The dye, in the presence of light, inactivated the enzyme and the inactivation was not dependent on pH. All the effects of the dye could be reversed by UMP, an allosteric inhibitor of the enzyme. The loss of enzyme activity on photoinactivation and the partial protection afforded by N-phosphonoacetyl-L-aspartate, a transition state analog and carbamyl phosphate plus succinate, a competitive inhibitor for aspartate, as well as the reversal of the dye difference spectrum by N-phosphonoacetyl-L-aspartate suggested that in the mung bean aspartate transcarbamylase, unlike in the case of *Escherichia coli* enzyme, the active and allosteric sites may be located close to each other.

**Keywords.** Mung bean; aspartate transcarbamylase; interactions; active site; rose bengal; allosteric

### Introduction

Dyes have been extensively used to probe the structure-function relationships of proteins (Glazer, 1970; Thompson *et al.*, 1975; Thompson and Stellwagen, 1976). The studies on the interaction of tetraiodofluorescein at the CTP and ATP binding site of *Escherichia coli* aspartate transcarbamylase (EC 2.1.3.2) provided valuable information on the regulatory mechanism of this enzyme (Jacobsberg *et al.*, 1975). Rose bengal (tetrachloro-tetraiodo fluorescein) was used to study the nature of nucleotide binding sites in some enzymes (Brand *et al.*, 1967; Bond *et al.*, 1970; Rippa *et al.*, 1970). It was also used to photoinactivate and identify the amino acid residues at the binding sites of some enzymes (Westhead, 1965; Kamogawa and Fukui, 1975; Coulson and Yonetani, 1972). Although UMP was the most potent allosteric effector of mung bean aspartate transcarbamylase, the enzyme was also inhibited by UDP and UTP (Savithri *et al.*, 1978a). In an earlier communication we have reported a modified procedure for the purification of the enzyme and have shown that the enzyme is probably regulated by

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Abbreviations used: PALA, N-Phosphonoacetyl-L-aspartate; PAGE, polyacrylamide gel electrophoresis.



slow association-dissociation between polymeric forms which respond differently to substrate(s) and allosteric effectors (Prasad and Appaji Rao, 1984). The present paper describes the interaction of rose bengal at the UMP binding region of mung bean aspartate transcarbamylase.

## Materials and methods

### Materials

All the chemicals except rose bengal and N-phosphonoacetyl-L-aspartate (PALA) were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Rose bengal was obtained from Eastman Kodak Co., Rochester, New York, USA. PALA was a kind gift from Dr. Leonard Kedda, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Maryland, USA.

### Methods

Aspartate transcarbamylase was purified as described earlier (Prasad, 1983; Prasad and Appaji Rao, 1984). The enzyme was assayed by a colorimetric procedure (Prescott and Jones, 1969). The standard assay mixture (1 ml) contained 0.1 M Tris-acetate buffer, pH 8.0; carbamyl phosphate, 5 mM; L-aspartate, 10 mM and an appropriate amount of the enzyme. The reaction was started by the addition of L-aspartate and terminated after 10 min of incubation at 25°C by the addition of 0.05 ml of 20% perchloric acid. The amount of N-carbamyl-L-aspartate formed was estimated by the method of Prescott and Jones (1969). When rose bengal was included in the assay mixture, all the incubations and the reaction were conducted in the dark to prevent photoinactivation of the enzyme by the dye. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1  $\mu$ mol of N-carbamyl-L-aspartic acid per min at 25°C and pH 8.0. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Rose bengal was purified by the method of Hoffee *et al.* (1967). Photooxidation of the enzyme in the presence of rose bengal was carried out in the electrode chamber of a Gilson KIC-oxygraph. The reaction vessel was surrounded by an outer jacket through which water was circulated at a constant temperature (26°C). The reaction mixture (0.7 ml) contained 20 mM Tris-acetate buffer, pH 8.0, rose bengal at the indicated concentrations and an appropriate amount of the enzyme. Photooxidation was initiated by switching on a 250 W Tungsten lamp placed at a distance of 6 cm from the reaction vessel. At various time intervals, aliquots (0.1 ml) were withdrawn and mixed with 0.8 ml of 0.1 M Tris-acetate buffer, pH 8.0 containing 2.5 mM carbamyl phosphate. The aliquots were stored in the dark at 4°C and assayed together at the end of the experiment for the residual enzyme activity by the addition of 10 mM L-aspartate. The activity at zero time was taken as 100 and the per cent residual activity at different periods of inactivation was calculated. The results were plotted in the form of a semi-logarithmic plot of  $\ln$  (per cent residual activity) versus time. The slopes of these lines, calculated by least square analysis gave the pseudo first order rate constant ( $k_{app}$ ) of inactivation at various concentrations of rose bengal.

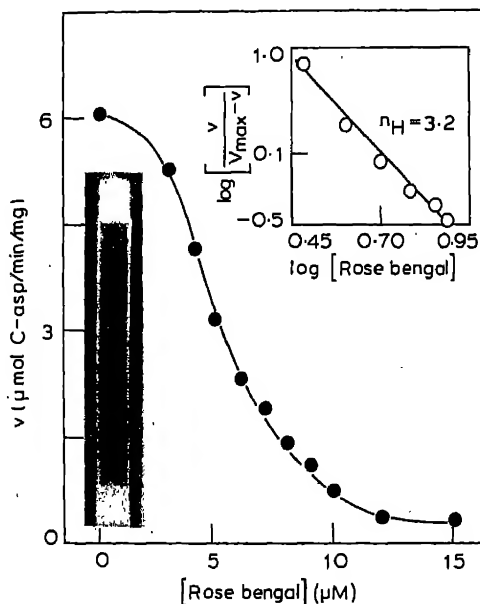
Absorption spectra were recorded at 25°C in a Cary 219 recording spectrophotometer using 0.3 ml quartz cells of 1 cm pathlength.

Polyacrylamide disc gel electrophoresis (PAGE) was conducted according to the method of Davis (1964) in 0.1 M Tris-glycine buffer, pH 8.6 containing 15  $\mu$ M rose bengal. The enzyme (40  $\mu$ g) was preincubated with 15  $\mu$ M rose bengal for 10 min in the dark and applied onto 7.5% gels. Electrophoresis was conducted in the dark at 4°C using a current of 2.5 mA per tube. Electrophoresis was terminated when the marker dye migrated to a distance of about 0.5 cm from the bottom of the gel. The gels were stained for protein by Coomassie Brilliant Blue R 250 and destained with methanol:acetic acid:water (43:7:50, v/v). Activity staining was carried out according to a slightly modified procedure of Grayson and Yon (1978). The gels were washed thoroughly with cold 0.1 M Tris-acetate buffer, pH 8.0 and incubated overnight in the same buffer containing 5 mM carbamyl phosphate, 10 mM L-aspartate and 10 mM  $\text{CaCl}_2$  at 4°C. Aspartate transcarbamylase activity was revealed as a white opalescent precipitate of calcium phosphate.

## Results

### *Inhibition of mung bean aspartate transcarbamylase activity by rose bengal*

The activity of the enzyme was inhibited by rose bengal in the dark at pH 8.0 in a sigmoidal fashion (figure 1) with a  $n_H$  value of 3.2 (figure 1, inset). From the Hill plot, a  $I_{0.5}^K$  of 5.6  $\mu$ M was calculated by a least squares analysis of the data.



**Figure 1.** Inhibition of the activity of the enzyme by rose bengal. *Inset:* Hill plot. The enzyme (2.6  $\mu$ g) was preincubated in the dark with various concentrations of rose bengal in the assay buffer for 10 min at 25°C followed by a second preincubation with 5 mM carbamyl phosphate. The enzyme (40  $\mu$ g) was preincubated in the dark with rose bengal and subjected to PAGE and the enzyme band was located by activity staining.

*Effect of rose bengal on carbamyl phosphate saturation of the enzyme*

Carbamyl phosphate saturation of the enzyme was determined at pH 8.0 in the absence and in the presence of 5  $\mu$ M rose bengal. The saturation of the enzyme in the absence of rose bengal was hyperbolic (figure 2). Maximum velocity of the reaction was attained at a carbamyl phosphate concentration of about 0.25 mM and increasing the substrate concentration beyond 0.25 mM had no effect on the velocity of the reaction. On the other hand, the enzyme showed a complex pattern of carbamyl phosphate saturation with a maximum and a plateau region in the presence of rose bengal. This pattern was similar to the carbamyl phosphate saturation pattern of a partially purified enzyme preparation when the reaction was started by the addition of the enzyme (Prasad and Appaji Rao, 1984).

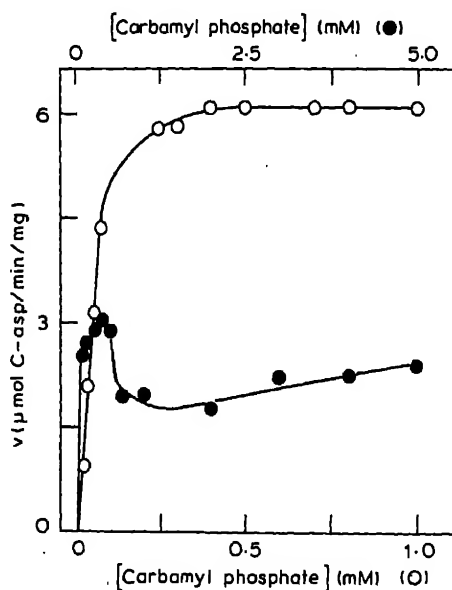


Figure 2. Effect of rose bengal on carbamyl phosphate saturation of the enzyme.

The enzyme (1.9  $\mu$ g) was preincubated with varying concentrations of carbamyl phosphate for 10 min (○) or with 5  $\mu$ M rose bengal for 10 min followed by varying concentrations of carbamyl phosphate (●). The reaction was started by the addition of 10 mM L-aspartate and the activity of the enzyme was determined as described.

*Effect of rose bengal on L-aspartate saturation of the enzyme*

Rose bengal acted as a noncompetitive inhibitor of the enzyme when L-aspartate was the varied substrate (figure 3). A replot of the slopes and intercepts (figure 3, inset) of the Lineweaver-Burk plots at different concentrations of rose bengal indicated that the dye was a linear noncompetitive inhibitor of the enzyme (Cleland, 1970). From the slope replot, a  $K_i$  value of 9  $\mu$ M was calculated for the dye.

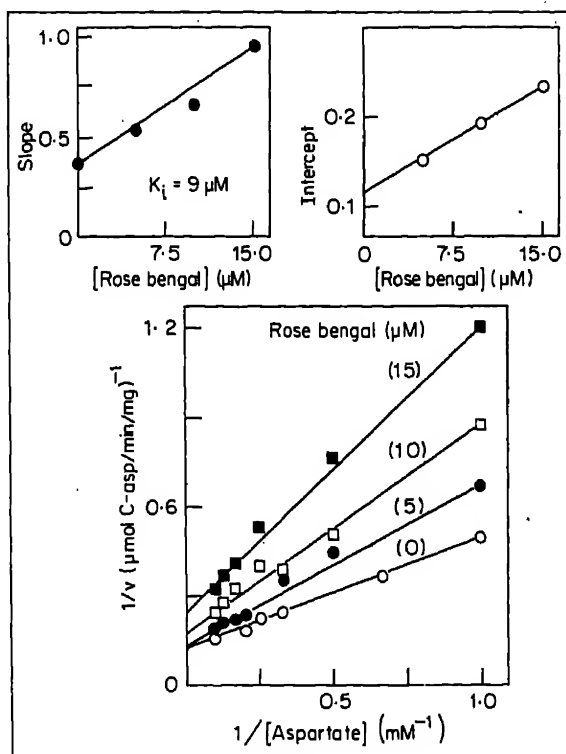


Figure 3. Lineweaver-Burk plots for L-aspartate saturation of the enzyme in the presence of 0 (○), 5 (●), 10 (□) and 15 μM (■) rose bengal.

The enzyme (1.7 μg) was preincubated in the dark with different fixed concentrations of rose bengal for 10 min in the assay buffer followed by a second preincubation with 5 mM carbamyl phosphate. Reaction was started by adding varying concentrations of L-aspartate. Inset: Replot of the slopes and intercepts of Lineweaver-Burk plots.

#### *Time course of the enzymatic reaction in the presence of rose bengal*

The kinetic effects of UMP and rose bengal was followed by monitoring the progress of the enzymatic reaction with time in the presence of rose bengal. The enzyme showed a linear progress curve in the absence of rose bengal (figure 4). When it was preincubated with 5 μM rose bengal and the reaction started by the addition of carbamyl phosphate plus L-aspartate, there was a lag phase in the time course of the reaction (figure 4). Preincubation with rose bengal followed by carbamyl phosphate (figure 4) abolished the rose bengal induced hysteretic behaviour. These results were similar to those observed with UMP (Prasad and Appaji Rao, 1984).

#### *Photoinactivation of the enzyme in the presence of rose bengal*

The photoinactivation of the enzyme in the presence of varying concentrations of rose bengal followed pseudo first order kinetics as indicated by the linear semilogarithmic plots (figure 5). Pseudo first order kinetics were observed until the enzyme was almost

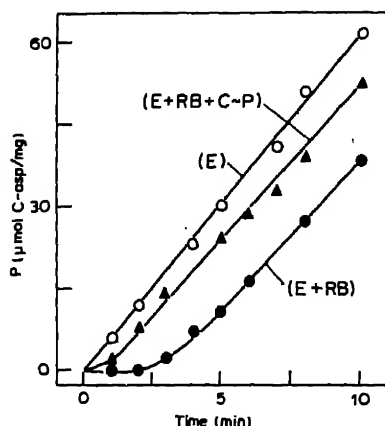


Figure 4. Time course of the reaction after preincubating the enzyme with rose bengal (●), rose bengal followed by carbamyl phosphate (▲) or with no preincubation (○).

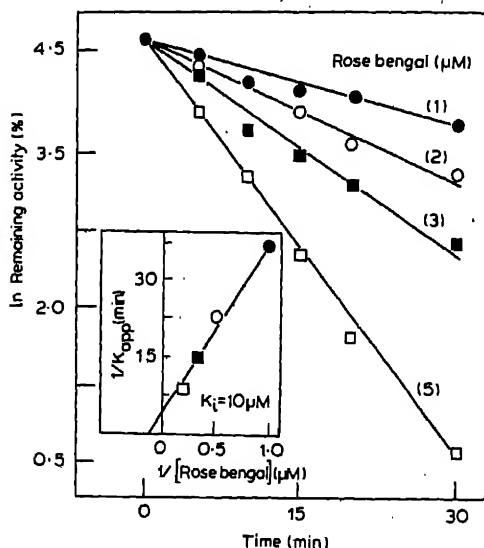


Figure 5. Photoinactivation of the enzyme in the presence of rose bengal. Semilogarithmic plots of  $\ln$  (% residual activity) vs time for the photo-inactivation of the enzyme in the presence of 1 (●), 2 (○), 3 (■) and 5  $\mu M$  (□) rose bengal. The enzyme (10  $\mu g$ ) was subjected to photooxidation in the presence of rose bengal as described under 'Methods'. Inset: Determination of the  $K_i$  for rose bengal.

completely inactivated. When  $(K_{app})^{-1}$  was plotted against  $[rose\ bengal]^{-1}$ , a linear relationship (correlation coefficient = 0.98) was obtained (inset, figure 5) indicating that photooxidation by rose bengal followed saturation kinetics. From a least square analysis of the data given in the figure 5, a  $K_i$  value of 10  $\mu M$  was obtained for rose bengal from the intercept on the X-axis which is in very good agreement with the  $K_i$  value of 9  $\mu M$  obtained from the inhibition studies (figure 3).

*Protection against photoinactivation in the presence of rose bengal by biospecific ligands*

In order to ascertain whether rose bengal was interacting at a specific site on the enzyme, the ability of biospecific ligands to protect the enzyme against photoinactivation of the enzyme by rose bengal was studied. The ability of various ligands to protect the enzyme is shown in figure 6 in the form of pseudo first order plots. Carbamyl phosphate (2.5 mM) and L-aspartate (10 mM) did not protect the enzyme against inactivation. The concentrations of these ligands were much higher than their  $K_m$  values (0.25 mM for carbamyl phosphate and 0.5 mM for L-aspartate). However, when succinate, a competitive inhibitor with respect to L-aspartate (Savithri *et al.*, 1978b) and carbamyl phosphate (2.5 mM) were present together, the enzyme was almost completely protected against photoinactivation. Similarly, the transition state analog, PALA (25  $\mu$ M) which has the structural features of both the substrates (Collins and Stark, 1971) or the allosteric effector, UMP (1 mM) also protected the enzyme against inactivation suggesting that blocking of either the allosteric site or both the substrate sites protected the enzyme against photoinactivation in the presence of rose bengal.

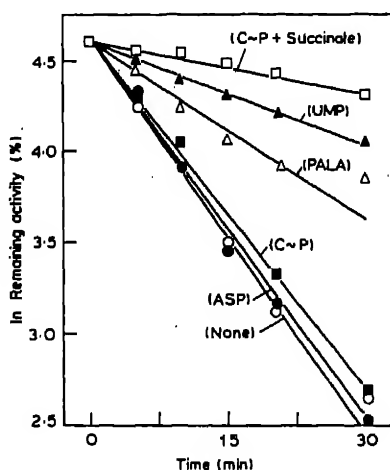


Figure 6. Protection by substrates and effectors against photoinactivation of the enzyme by rose bengal.

The enzyme (10  $\mu$ g) was subjected to photoinactivation in the presence of 3  $\mu$ M rose bengal as described. The inactivation mixture also contained carbamyl phosphate (2.5 mM) or L-aspartate (10 mM) or carbamyl phosphate (2.5 mM) plus succinate (10 mM) or PALA (25  $\mu$ M) or UMP (1 mM) or no ligand added.

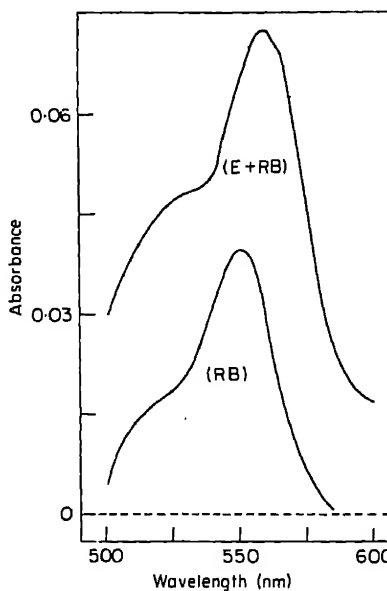
*Effect of pH on the rate of photoinactivation of the enzyme*

It was earlier reported (Westhead, 1965) that the rate of photooxidation of histidine in the presence of rose bengal was pH dependent with the unprotonated form being more susceptible to photooxidation. To examine the possibility whether the inactivation of the enzyme by rose bengal in the presence of light was due to the loss of a histidine residue, the rate of photoinactivation of the enzyme was studied at pH 6.0, 7.0 and 8.0.

The  $K_{app}$  value at these pH values was  $0.015 \text{ min}^{-1}$ . As histidine has a  $pK_a$  of 6–7 in most proteins (Tanford, 1962), this observation suggests that the loss of enzyme activity by photooxidation may probably be not due to the loss of a histidine residue. Attempts to demonstrate the presence of a reactive histidine by chemical modification of the enzyme were unsuccessful suggesting that this amino acid may not be essential for activity.

### *Spectral studies on the interaction of rose bengal with the enzyme*

**Effect of enzyme on the rose bengal absorption spectrum:** Rose bengal showed an absorption maximum at 550 nm (figure 7) in 10 mM potassium phosphate buffer, pH 7.6. Upon the addition of the enzyme, the absorption maximum of the dye showed a red shift of 7 nm in its absorption along with an increase in absorbance at all wavelengths (figure 7). The dye spectrum showed a similar red shift and an increase in absorbance when it was recorded in 50% ethanol (data not shown).



**Figure 7.** Effect of the addition of the enzyme on the absorption spectrum of the dye.

The absorption spectrum of rose bengal ( $0.42 \mu\text{M}$ ) was recorded in 10 mM potassium phosphate buffer, pH 7.6 from 500 to 600 nm. The spectrum of the dye ( $0.42 \mu\text{M}$ ) was again recorded under similar conditions in the presence of the enzyme ( $2.5 \mu\text{M}$ ).

**Difference spectra of the dye in the presence of the enzyme and the enzyme plus ligands:** The difference spectrum of the dye (450 to 570 nm) upon the addition of the enzyme is shown in figure 8. The difference spectrum showed a peak around 565 nm and a trough at 540–550 nm. When UMP ( $2.7 \text{ mM}$ ) was added to the reference cuvette containing the dye and to the sample cuvette containing the dye-enzyme complex, there was a perturbation of the difference spectrum (figure 8A). On the other hand, PALA ( $200 \mu\text{M}$ ) had no effect on the difference spectrum of the dye and the dye-enzyme complex (figure 8B). However, when UMP was added to the sample cuvette containing

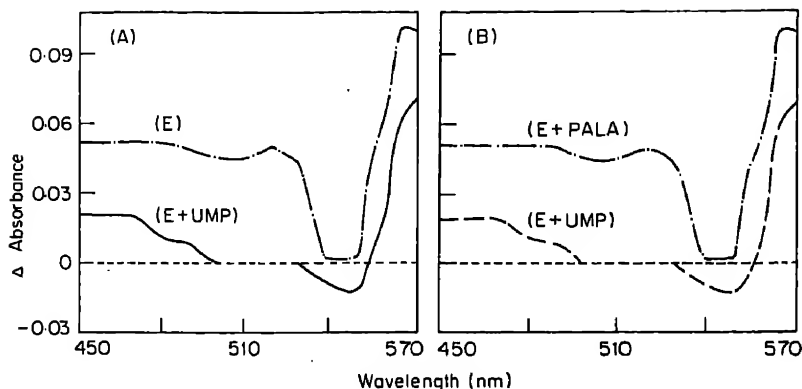


Figure 8. A. Difference spectrum of the dye in the presence of enzyme (E) and enzyme + UMP (E + UMP).

Rose bengal ( $2.1 \mu\text{M}$ ) was taken in both the sample and reference cells in 10 mM potassium phosphate buffer, pH 7.6 and the base line was recorded from 450 to 570 nm. The sample cuvette was made up with dye ( $2.1 \mu\text{M}$ ) and enzyme ( $40 \mu\text{g}$ ) in the same buffer and the difference spectrum was recorded. UMP ( $2.7 \text{ mM}$ ) was added to both the cells and the spectrum was again recorded.

B. Difference spectrum of the dye in the presence of (E + PALA) or (E + PALA + UMP).

The difference spectrum of the dye ( $2.1 \mu\text{M}$ ) was recorded after the addition of enzyme ( $40 \mu\text{g}$ ) as described above. PALA ( $200 \mu\text{M}$ ) was added to both the cells and the difference spectrum was re-recorded. UMP ( $2.7 \text{ mM}$ ) was added to the reference cell containing the dye + PALA and to the sample cell containing the dye + enzyme + PALA and the difference spectrum was again recorded.

the dye-enzyme-PALA complex and to the reference cuvette containing the dye and PALA, the difference spectrum was perturbed in a manner similar to that observed in figure 8A. This result showed that only UMP, but not PALA could displace the dye from the enzyme.

#### *Electrophoretic behaviour of the enzyme in the presence of rose bengal*

The purified mung bean aspartate transcarbamylase exhibited multiple bands on PAGE when stained both for protein and activity. UMP converted this multiple band pattern on PAGE to a single band (Prasad and Appaji Rao, 1984). To further examine the similarities in the effects of rose bengal and UMP on the mung bean enzyme, it was subjected to PAGE in the presence of  $15 \mu\text{M}$  rose bengal. As seen in figure 1 (inset), the enzyme migrated as a single band in the presence of rose bengal as revealed by activity staining.

#### Discussion

The structure-function relationships of proteins have been probed by a variety of methods. The results presented in this paper demonstrate that simple kinetic measurements can be used to locate the allosteric site on mung bean aspartate transcarbamylase using the interaction of rose bengal as a convenient probe. The



following experimental observations presented in the paper suggest that rose bengal was interacting at the UMP binding domain of the enzyme: (i) The inhibition patterns of the dye in the absence of light (figure 1) and of UMP (Prasad and Appaji Rao, 1984) were sigmoidal with  $n_H$  value of 3.2 and 2.2, respectively. (ii) Rose bengal altered the electrophoretic behaviour of the enzyme, converting the multiple enzymatically active bands into a single band (figure 1, inset) in a manner similar to that by UMP (Prasad and Appaji Rao, 1984). (iii) The absorption spectrum of the dye was perturbed upon binding to the enzyme (figure 7). The red shift in the visible absorption spectrum of the dye and the increase in absorbance upon the addition of the enzyme suggested that the dye was probably binding in a hydrophobic region on the enzyme. Similar observations were made with the interaction of rose bengal with RNA polymerase (Wu and Wu, 1973a). Additional evidence for the dye binding in a non-polar region on the enzyme was the observation that the dye spectrum showed a similar red shift and an increase in absorbance in 50% ethanol (data not shown). The dye difference spectrum was perturbed by UMP (figure 8A), but not by PALA (figure 8B) further suggesting that the dye was probably interacting at the UMP binding domain on the enzyme. (iv) Both the dye (figure 3) and UMP (Savithri *et al.*, 1978a) acted as noncompetitive inhibitors of the enzyme when L-aspartate was the varied substrate. (v) Both rose bengal (figure 5) and UMP (Prasad and Appaji Rao, 1984) induced hysteresis in the enzyme which was abolished by preincubation with carbamyl phosphate. (vi) Finally, UMP protected the enzyme against photoinactivation in the presence of rose bengal (figure 6).

The interaction of rose bengal at the UMP binding domain might appear surprising as this dye does not bear any structural resemblance to nucleoside- mono, -di or -triphosphate. However, it is pertinent to point out that Glazer (1970) has suggested that the binding of dyes to proteins occurs in regions overlapping the binding sites for substrates, coenzymes and prosthetic groups in preference to other regions on the protein surface. This preferential binding might reflect the special stereochemical features of the biospecific ligand binding sites and their hydrophobicity relative to other regions. Although, Cibacron Blue F3GA was originally believed to interact at the dinucleotide fold (Thompson *et al.*, 1975; Thompson and Stellwagen, 1976; Stellwagen, 1977), extensive literature is now available to indicate that the dye might interact at other regions on some proteins (Steitz *et al.*, 1976; Chambers and Dunlap, 1979; Ramesh and Appaji Rao, 1980). Rose bengal was shown to bind at the pyridine nucleotide binding sites in some dehydrogenases (Brand *et al.*, 1967; Bond *et al.*, 1970; Rippa *et al.*, 1970). Interaction of rose bengal with RNA polymerase (Wu and Wu, 1973a, b) altered the catalytic properties of the enzyme markedly without binding to the nucleoside triphosphate or the DNA template binding sites. Thus, it is clear that a wide range of interactions of the dyes with proteins is possible.

An interesting suggestion that can be made from these studies is that in mung bean aspartate transcarbamylase the active and the allosteric sites are probably close to each other. The following arguments are compatible with these suggestions: (i) The dye which binds at the UMP binding domain caused inactivation of the enzyme in the presence of light (figure 5). This result suggested that the loss of the allosteric site had adverse effect on the catalytic activity of the enzyme. The enzyme was protected against photoinactivation both by UMP and PALA (figure 6). This result showed that blocking of either both the substrate sites or the allosteric site protected the enzyme.

(ii) The allosteric and the active sites were distinct as suggested by the observation that the difference spectrum of the dye was perturbed by UMP, but not by PALA (figure 8). In the case of *E. coli* aspartate transcarbamylase, the regulatory site could be destroyed without affecting the catalytic activity (Gerhart, 1970) showing that the active site and allosteric site were spatially well separated. In the mung bean enzyme, it may be postulated that due to the nearness of both the sites, the destruction of the allosteric site adversely affected the active site. Mung bean enzyme is a hexamer with identical subunits (Prasad and Appaji Rao, 1983) unlike the *E. coli* enzyme which has separate catalytic and regulatory subunits (Gerhart, 1970). It could be argued that rose bengal has two binding sites, one for inhibition and the other for photoinactivation. But the nearly identical  $K_i$  values of 9 and 10  $\mu\text{M}$  obtained from the inhibition (figure 3) and the inactivation (figure 6) argued against such a possibility. The observation of saturation kinetics in photoinactivation and the finding that pseudo first order kinetics were observed until the complete loss of activity suggested that rose bengal was interacting at a specific site on the enzyme. The inability to desensitize the enzyme without affecting the catalytic activity by other physical and chemical means (data not shown) also lend support to the hypothesis that in mung bean aspartate transcarbamylase, the active and the allosteric sites might be close to each other. The intermediary plateau region in carbamyl phosphate saturation upon preincubating the enzyme with the dye may be related to the ability of the dye to induce hysteresis (figure 4) and cause aggregation of the enzyme similar to UMP (Prasad and Appaji Rao, 1984). A partially purified enzyme preparation gave similar carbamyl phosphate saturation curves (Prasad and Appaji Rao, 1983) which was consistent with a slow association-dissociation of the enzyme (Kurganov *et al.*, 1976).

All the above results are consistent with the hypothesis that the dye binds in a hydrophobic region on the enzyme which overlaps the regulatory site.

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## Studies on the conformations of sialyloligosaccharides and implications on the binding specificity of neuraminidases

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**Abstract.** Theoretical investigations, using semi-empirical potential functions have been carried out to predict the favoured conformations of the terminal dissaccharide fragments of various sialyloligosaccharides. The proposed conformational similarity for these fragments has been correlated to the binding specificity of neuraminidases. These calculations predict that bacterial neuraminidases have a binding site which can accommodate only two sugar residues and virus neuraminidases have a binding site which can accommodate more than two sugar residues.

**Keywords.** Conformation: N-acetylneuraminic acid; neuraminidases; sialic acid; sialyloligosaccharides.

### Introduction

Sialic acid (N-acetylneuraminic acid - NeuNAc) which is an important constituent of many of the cell surface carbohydrates plays a dominant role in the biological functions of cells (Jeanloz and Codington, 1976; Schauer, 1982). It mainly occurs as the terminal sugar residue in glycoconjugates. The predominant linkages that have been observed for sialic acid (SA) in glycoconjugates are

SA $\alpha$ (2-3)Gal-R

SA $\alpha$ (2-3)GalNAc-R

SA $\alpha$ (2-6)Gal-R

SA $\alpha$ (2-6)GalNAc-R

SA $\alpha$ (2-8)SA-R

where R is the carbohydrate core structure. Neuraminidase enzymes of bacterial origin (*Vibrio cholerae* neuraminidase, *Clostridium perfringens* neuraminidase and *Arthrobacter ureafaciens* neuraminidase) are widely used reagents for cleaving the SA from sialyloligosaccharides. These enzymes are nonspecific for the type of sialyloligosaccharides which serve as substrates (Uchida *et al.*, 1979; Corfield *et al.*, 1981, 1983), i.e. their activity does not depend on whether the SA (NeuNAc) is linked to the C3 of Gal or GalNAc, C6 of Gal or GalNAc or C8 of another SA (NeuNAc). On the other hand, virus neuraminidases (Newcastle disease virus, Fowl plague virus and Influenza A<sub>2</sub> virus neuraminidases) cleave 2  $\rightarrow$  3 but not 2  $\rightarrow$  6 (Schauer, 1982; Paulson *et al.*, 1982) linkages. Newcastle disease virus neuraminidase is able to cleave 2  $\rightarrow$  8 linkages

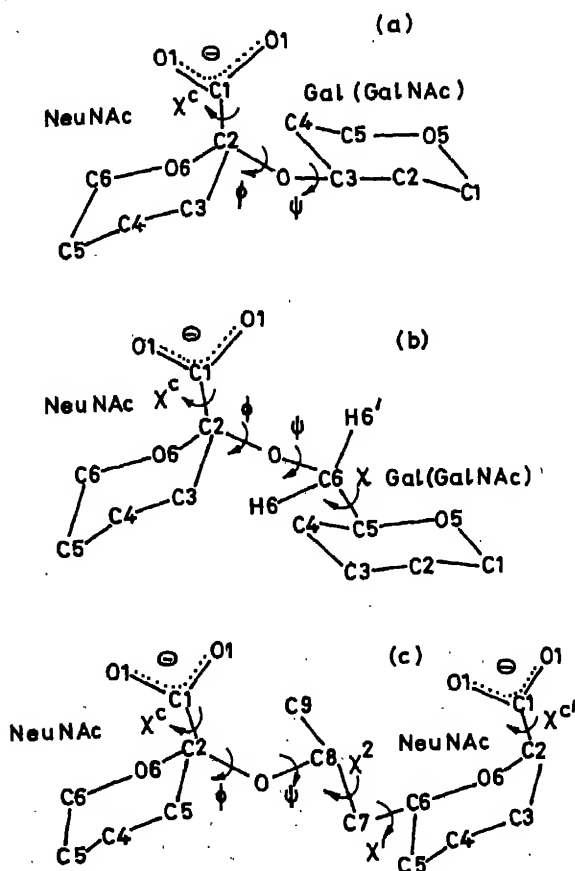
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Abbreviations used: NeuNAc, N-Acetylneuraminic acid; SA, sialic acid.

whereas Fowl plague virus neuraminidase and Influenza A<sub>2</sub> virus neuraminidase do not. To understand the binding specificity of neuraminidase enzymes on various substrates (sialyloligosaccharides) an attempt has been made using semi-empirical potential functions to study the favoured conformations of the terminal disaccharide fragments of the sialyloligosaccharides.

### Method of calculation

The various disaccharide fragments that were studied are shown in figure 1. Steric maps were constructed for all the disaccharide fragments using the contact criteria (Ramachandran and Sasisekharan, 1968). Various conformations were generated by allowing rotations about the interunit glycosidic bond C2-O ( $\phi$ -rotation) and O-CX ( $\psi$ -rotation) from  $-180^\circ$  to  $+180^\circ$  at intervals of  $10^\circ$ . All the sugar residues were



**Figure 1.** Numbering of atoms and possible rotational angles of the disaccharide fragment. (a) NeuNAc(2-3)Gal (NeuNAc(2-3)GalNAc), (b) NeuNAc(2-6)Gal (NeuNAc(2-6)GalNAc), (c) NeuNAc(2-8)NeuNAc. The side groups are not shown.

assumed to be in  ${}^4C_1(D)$  chair form except NeuNAc which was assumed to be in  ${}^2C_5(D)$  chair form. The atomic coordinates used for these residues were based on the geometry of Arnott and Scott (1972). The acetamido group of N-acetylgalactosamine was fixed using Pauling-Corey geometry (Corey and Pauling, 1953) so that C2-H2 and N-H bonds were trans. The geometry used for the NeuNAc was the same as described earlier (Veluraja and Rao, 1980, 1983, 1984). The side chains of NeuNAc (glycerol side chain and N-acetamido group) were fixed in their respective minimum energy conformations (Veluraja and Rao, 1980). The bond angles at the glycosidic oxygen atoms were fixed at the average value of  $117.5^\circ$ . The initial conformations were defined as

$\phi = 0$  when C1-C2 *cis* to O-CX

$\psi = 0$  when C2-O *cis* to CX-HX

$\chi^c = 0$  when O1-C1 *cis* to C2-O6

$\chi = 0$  when O-C6 *cis* to C5-O5

$\chi^1 = 0$  when C5-C6 *cis* to C7-C8

$\chi^2 = 0$  when C6-C7 *cis* to C8-C9

(where X represents the number of the carbon atom involved in the glycosidic linkage). In NeuNAc  $\alpha(2-6)$ Gal (NeuNAc $\alpha(2-6)$  GalNAc) H6 is that hydrogen which makes an angle of  $\text{H6-C6-C5-O5} = 120^\circ$  when  $\chi = 0^\circ$ . Clockwise rotation is taken as positive.

Steric maps for the disaccharide fragment NeuNAc $\alpha(2-8)$ -NeuNAc were obtained by fixing  $\chi^1$  and  $\chi^2$  in all the staggered positions. The steric map obtained when  $\chi^1$  and  $\chi^2$  were fixed in the minimum energy conformation ( $\chi^1 = 150^\circ$ ,  $\chi^2 = 70^\circ$ ) alone is shown in figure 2.

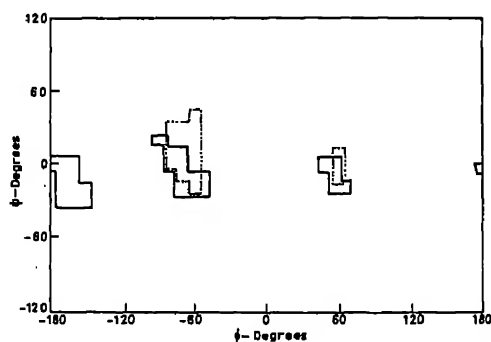


Figure 2. Steric map for the disaccharide fragment (—) NeuNAc $\alpha(2-3)$ Gal (.....) NeuNAc $\alpha(2-8)$ NeuNAc ( $\chi^1 = 150^\circ$ ;  $\chi^2 = 70^\circ$ ).

The potential energy of the molecules was computed taking into account the nonbonded, electrostatic and torsional contributions. The nonbonded contribution was computed using the modified Lennard-Jones potential functions and the constants given by Momany *et al.* (1975). The  $\sigma$ -charge on the various atoms of the ionized form of NeuNAc (the form which is present at physiological pH) was calculated following MO-LCAO (Molecular Orbital-Linear Combination of Atomic Orbitals) method of

Del Re *et al.* (1963).  $\pi$ -Charges for the acetamido group and carboxylic acid group were taken from Vijayalakshmi (1972). Since the carboxylic acid group was assumed to be in the ionized state,  $\pi$ -charge was distributed equally on both the oxygen atoms. The total charge on each atom was obtained by summing  $\sigma$ - and  $\pi$ -charges. For the carboxylic oxygen atoms an additional charge of about  $-0.5$  units was added as suggested by Del Re *et al.* (1963). The net charge associated with each atom was used to compute electrostatic energy.

The disaccharide fragments can assume a large number of conformations, due to possible rotations about the interunit bonds. Hence the energy was minimized as a function of rotational angles following the minimization procedure of Fletcher and Powell (1963) and Davidon (1959).

### Results and discussion

The steric maps for the disaccharides NeuNAc $\alpha$ (2-3)Gal, NeuNAc $\alpha$ (2-8)NeuNAc and NeuNAc $\alpha$ (2-6)Gal, (figures 2 and 3) indicate that less than 4% of the total region is allowed in the  $(\phi, \psi)$  plane in agreement with earlier results (Sathyanarayana and Rao, 1971). The addition of the acetamido group at the C2 position of the galactose residue does not appreciably affect the allowed region in the  $(\phi, \psi)$  plane of the NeuNAc $\alpha$ (2-3)Gal and NeuNAc $\alpha$ (2-6)Gal. Therefore the steric maps for the disaccharide fragments NeuNAc $\alpha$ (2-3)GalNAc and NeuNAc $\alpha$ -(2-6)GalNAc are not shown.

In the disaccharide fragments NeuNAc $\alpha$ (2-3)Gal and NeuNAc $\alpha$ (2-3)GalNAc,  $(\phi, \psi)$  favour values around  $(-70^\circ, -10^\circ)$  (tables 1 and 2). When they favour values around

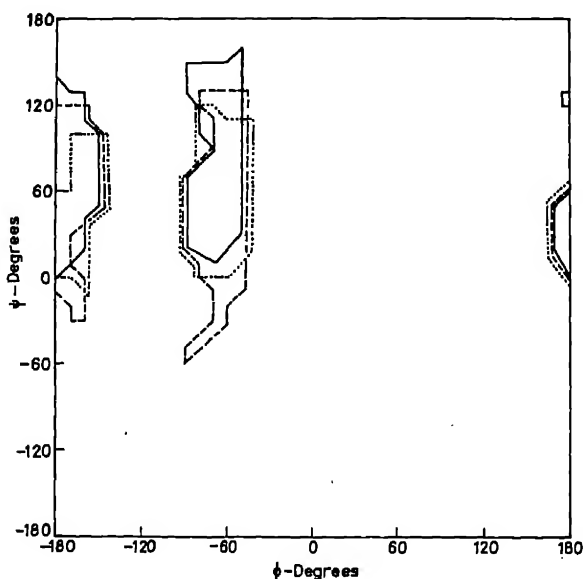


Figure 3. Steric map for the disaccharide fragment NeuNAc $\alpha$ (2-6)Gal. (—) ( $\chi = 180^\circ$ ); (---) ( $\chi = 60^\circ$ ); (.....) ( $\chi = -60^\circ$ ).

**Table 1.** Minimum energy conformations of NeuNAc $\alpha$ (2-3)Gal.

$\chi^c$	$\phi$	$\psi$	Relative energy kcal mol <sup>-1</sup>
-35	-67	-7	0.0
-38	-150	-20	4.0
-42	55	-8	7.9

**Table 2.** Minimum energy conformations of NeuNAc $\alpha$ (2-3)GalNAc.

$\chi^c$	$\phi$	$\psi$	Relative energy kcal mol <sup>-1</sup>
-35	-69	-6	0.0
-41	56	-10	4.7
-38	-154	-21	6.3

(-155°, -20°) and (55°, -10°) the energy increases by more than 4 kcal mol<sup>-1</sup>. This rules out the possibility of occurrence of these disaccharide fragments in these conformations. Projections of the global minimum energy conformers of NeuNAc $\alpha$ (2-3)Gal and NeuNAc $\alpha$ (2-3)GalNAc are shown in figures 4a and 5a.

Tables 3 and 4 give the probable conformers of the disaccharide fragments NeuNAc $\alpha$ (2-6)Gal and NeuNAc $\alpha$ (2-6)GalNAc respectively. It is interesting to note

**Table 3.** Minimum energy conformations of NeuNAc $\alpha$ (2-6)Gal.

$\chi^c$	$\phi$	$\psi$	$\chi$	Relative energy kcal mol <sup>-1</sup>
-35	-66	-13	73	0.0
-35	-80	144	-176	0.9
-36	-82	34	65	1.0
-34	-61	80	180	1.5
-33	-68	60	63	1.5
-34	-83	52	177	1.6
-37	-147	70	178	2.5
-39	-171	17	175	2.9
-35	-61	86	-53	3.2
-38	-148	-28	83	3.3
-35	-74	130	70	3.3
-32	-141	67	69	3.4
-44	64	84	-177	4.0



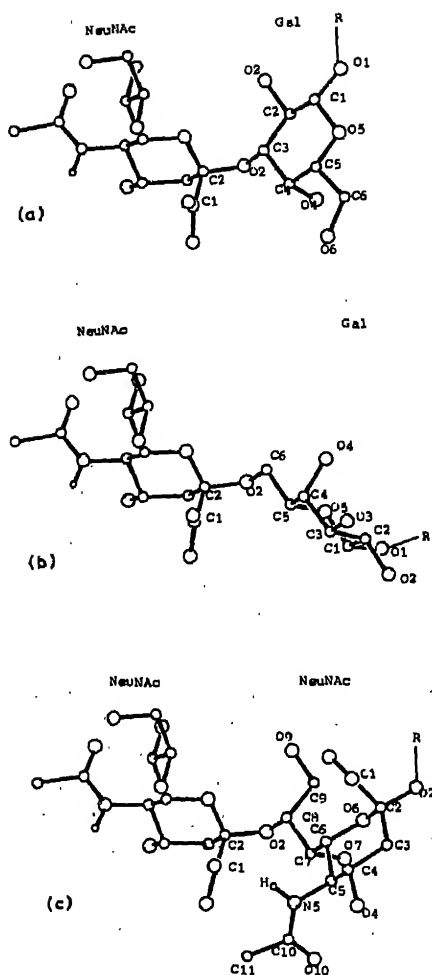


Figure 4. Projection of the global minimum energy conformer of (a) NeuNAc $\alpha$ (2-3)Gal, (b) NeuNAc $\alpha$ (2-6)Gal and (c) NeuNAc $\alpha$ (2-8)NeuNAc.

that both the disaccharide fragments favour similar conformations (conformers 1 to 4 of table 3 are equivalent to conformers 1 to 4 of table 4). The dihedral angles ( $\phi$ ,  $\psi$ ,  $\chi$ ) which define the conformation of these disaccharide fragments favour values around ( $-70^\circ$ ,  $-10^\circ$ ,  $70^\circ$ ). When they favour values around ( $-80^\circ$ ,  $145^\circ$ ,  $-175^\circ$ ); ( $-80^\circ$ ,  $40^\circ$ ,  $65^\circ$ ) or ( $-60^\circ$ ,  $80^\circ$ ,  $180^\circ$ ) the conformational energy increases by about 0.9, 1.0 and 1.5 kcal mol $^{-1}$  respectively in NeuNAc $\alpha$ (2-6)Gal and 1.6, 1.9 and 2.7 kcal mol $^{-1}$  respectively in NeuNAc $\alpha$ (2-6)GalNAc. This indicates that the addition of the acetamido group at C2 of galactose in NeuNAc $\alpha$ (2-6)Gal to give NeuNAc $\alpha$ (2-6)GalNAc affects neither the favoured conformations nor the relative energies of these molecules significantly. Projections of the global minimum energy conformers of NeuNAc $\alpha$ (2-6)Gal and NeuNAc $\alpha$ (2-6)GalNAc are shown in figures 4b and 5b.

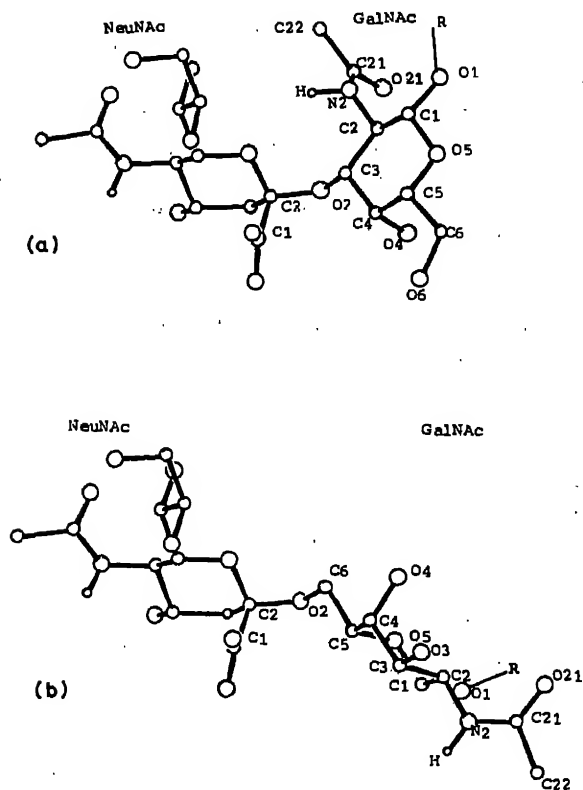


Figure 5. Projection of the global minimum energy conformer of (a) NeuNAc $\alpha$ (2-3)GalNAc and (b) NeuNAc $\alpha$ (2-6)GalNAc.

Table 4. Minimum energy conformations of NeuNAc $\alpha$ (2-6)GalNAc

$\chi^e$	$\phi$	$\psi$	$\chi$	Relative energy kcal mol <sup>-1</sup>
-35	-66	-14	73	0.0
-35	-81	145	-176	1.6
-32	-85	48	64	1.9
-36	-60	87	180	2.7
-34	-84	52	175	2.9
-38	-148	70	177	3.4
-38	-146	-29	85	3.5
-39	-170	14	172	3.7
-39	-142	67	70	4.1

In the disialic acid fragment NeuNAc $\alpha$ (2-8)NeuNAc the dihedral angles ( $\phi, \psi, \chi^2, \chi^1$ ), which define its conformation prefer values around ( $-75^\circ, 10^\circ, 70^\circ, 150^\circ$ ) (table 1). When these dihedral angles assume values around ( $-130^\circ, 45^\circ, 160^\circ, 115^\circ$ ) or ( $-160^\circ, 0^\circ, 80^\circ, 170^\circ$ ) the conformational energy increases by about 3.4 and 4.6 kcal mol $^{-1}$  respectively. This suggests the low probability of occurrence of the disialic acid fragment in a conformer other than the global minimum energy conformer. The projection of the low energy conformer of NeuNAc $\alpha$ (2-8)NeuNAc is shown in figure 4c.

Table 5. Minimum energy conformations of NeuNAc $\alpha$ (2-8)NeuNAc.

$\chi^c$	$\phi$	$\psi$	$\chi^2$	$\chi^1$	$\chi^{c'}$	Relative energy kcal mol $^{-1}$
-39	-76	8	70	148	-46	0.0
-42	-128	43	160	114	-36	3.4
-39	-165	-2	86	169	-44	4.6

A comparison of the minimum energy conformers of the disaccharide fragments NeuNAc $\alpha$ (2-3)Gal, NeuNAc $\alpha$ (2-6)Gal and NeuNAc $\alpha$ (2-8)NeuNAc (tables 1, 3 and 4 and figure 4) together with the geometry of the second residues show that the following set of dihedral angles

$\phi, \psi$ , O2-C3-C4-C5 and C3-C4-C5-C6 (approximately  $-70^\circ, -10^\circ, 180^\circ$  and  $180^\circ$ ) in NeuNAc $\alpha$ (2-3)Gal,

$\phi, \psi$ , O2-C6-C5-C4 ( $\chi + 120^\circ$ ) and C6-C5-C4-C3 (approximately  $-70^\circ, -10^\circ, -170^\circ$  ( $\chi = 70^\circ$ ) and  $180^\circ$ ) in NeuNAc $\alpha$ (2-6)Gal

and

$\phi, \psi$ , O2-C8-C7-C6 ( $\chi^2 + 120^\circ$ ) and C8-C7-C6-C5 ( $\chi^1$ )

(approximately  $-70^\circ, -10^\circ, -170^\circ$  ( $\chi^2 = 70^\circ$ ) and  $150^\circ$ ) in NeuNAc $\alpha$ (2-8) NeuNAc favour approximately similar values. In other words, when the first residue (NuNAc) of these disaccharide fragments are superimposed then the fragment

O2-C3-C4-C5-C6 of Gal of NeuNAc $\alpha$ (2-3)Gal,

O2-C6-C5-C4-C3 of Gal of NeuNAc $\alpha$ (2-6)Gal and

O2-C8-C7-C6-C5 of second NeuNAc of NeuNAc $\alpha$ (2-8)NeuNAc

can take up similar orientations. It may be due to this conformational similarity, that the neuraminidase enzymes are able to cleave NeuNAc residue from the sialyloligosaccharide differing in the types of linkages. It is also interesting to note that the oxygen atoms O4, O5 of the galactose residue of NeuNAc $\alpha$ (2-3)Gal, O5, O4 of the galactose residue of NeuNAc $\alpha$ (2-6)Gal and O7, O6 of the second NeuNAc residue of NeuNAc $\alpha$ (2-8)NeuNAc also assume similar orientations. There is thus, a possibility that these atoms may be involved in hydrogen bond formation with the active site of the neuraminidase enzymes.

As mentioned earlier, in the disaccharide fragments NeuNAc $\alpha$ (2-6)Gal and NeuNAc $\alpha$ (2-8)NeuNAc the C3 atom of Gal and the C5 atom of the second NeuNAc residue occupy similar positions. However, the electronegative atoms (O3 of Gal and N5 of NeuNAc) which are attached to them differ in their orientation and are fixed because of the ring geometry. In the disaccharide fragment NeuNAc $\alpha$ (2-3)Gal, the C6 atom of galactose also occupies the same position as that of the two carbon atoms mentioned above. The electronegative atom (O6) which is attached to it can occupy all the three staggered orientation because of the possible freedom of rotation around an exocyclic bond. It is, therefore, possible for this electronegative atom (O6) to occupy, in one orientation a position similar to that of O3 in NeuNAc $\alpha$ (2-6)Gal and in another orientation a position similar to the N5 in NeuNAc $\alpha$ -(2-8)NeuNAc. Thus, if the O3 of NeuNAc $\alpha$ (2-6)Gal is involved in an interaction (hydrogen bond formation) with a neuraminidase enzyme then the O6 of NeuNAc $\alpha$ (2-3)Gal can give the same type of interaction but the N5 atom of NeuNAc $\alpha$ (2-8)NeuNAc can not. If on the other hand the N5 atom of NeuNAc $\alpha$ (2-8)NeuNAc interacts with a neuraminidase enzyme (hydrogen bond formation) then the O6 of NeuNAc $\alpha$ (2-3)Gal can give the same type of interaction whereas the O3 of NeuNAc $\alpha$ (2-6)Gal can not.

It is also interesting to note that in NeuNAc $\alpha$ (2-3)Gal the O2 hydroxyl of the galactose residue (fixed because of the ring geometry) and the O9 hydroxyl (three staggered orientations are possible because of the rotation around exocyclic bond) of the second NeuNAc residue in NeuNAc $\alpha$ (2-8)NeuNAc can also occupy similar position. No corresponding electronegative atom is present in that position in NeuNAc $\alpha$ (2-6)Gal. A bulky group (acetamido group) is present on the second NeuNAc residue in NeuNAc $\alpha$ (2-8)NeuNAc. While no bulky group is present in that position in the other two disaccharide fragments (figure 4), when the third sugar residue (R) is attached to these three disaccharide fragments there will be some differences in the overall shape of these molecules because of the differences in the orientation of the anomeric oxygens. The third sugar residue is positioned approximately in the same direction in NeuNAc $\alpha$ (2-3)Gal and NeuNAc $\alpha$ (2-8)NeuNAc and will be different in NeuNAc $\alpha$ (2-6)Gal (figure 4). This would mean that the orientation of the disaccharide fragment of the latter with respect to the rest of the sugar residues in the carbohydrate chain will be different from that of the former two disaccharides. These results suggest that if the neuraminidase enzymes accommodate only two sugar residues (terminal disaccharide fragments) in its binding site, because of the conformational similarity already mentioned all the three substrates should show comparable activity. On the other hand, if the binding site accommodates more than two sugar residues, then the enzymes should show comparable binding activity with NeuNAc $\alpha$ (2-3)Gal and NeuNAc $\alpha$ (2-8)NeuNAc but not with NeuNAc $\alpha$ (2-6)Gal because of differences in the orientation of the terminal disaccharide fragments with respect to the rest of the carbohydrate chain.

Thus neuraminidase enzymes of bacterial origin (*Clostridium perfringens*, *Vibrio cholerae*, *Arthrobacter ureafaciens*) which show comparable binding activity with all the three substrates may be able to accommodate not more than two sugar residues in their binding sites. The slight differences in their activity can be accounted for, by the difference in the orientation of some of the side group as mentioned earlier. Neuraminidase enzymes from viruses (Newcastle disease virus, Fowl plague virus,

Influenza A<sub>2</sub> virus) which show very good activity with NeuNAc $\alpha$ (2-3)Gal and negligible activity with NeuNAc $\alpha$ (2-6)Gal containing sialyloligosaccharides, may have large binding site which accommodate more than two sugar residues. As has been mentioned earlier, if the binding site is large, these enzymes should show comparable activity with NeuNAc $\alpha$ (2-8)NeuNAc containing sialyloligosaccharides also. Newcastle disease virus neuraminidase shows good activity with NeuNAc $\alpha$ (2-8)NeuNAc containing sialyloligosaccharides (Schauer, 1982) in agreement with theoretical predictions. Fowl plague virus neuraminidase and influenza A<sub>2</sub> virus neuraminidase show poor activity with NeuNAc $\alpha$ (2-8)NeuNAc containing sialyloligosaccharides. This may be due to the presence of the bulky acetamido group at C5 position of the second NeuNAc residue (figure 4) which may be causing steric problems with the combining site of these neuraminidase enzymes.

As mentioned earlier, the substitution of an acetamido group at C2 position of the galactose residue of NeuNAc $\alpha$ (2-3)Gal and NeuNAc $\alpha$ (2-6)Gal does not affect the favoured minimum energy conformations of these disaccharides (tables 1 to 4 and figures 4 and 5). These disaccharides (NeuNAc $\alpha$ (2-3)GalNAc, NeuNAc $\alpha$ (2-6)GalNAc) are also cleaved by the neuraminidase enzymes indicating that the acetamido group at the C2 position of the galactose residue does not hinder the binding of the neuraminidase enzymes.

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## Location of valinomycin in lipid vesicles

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**Abstract.** The location of the cyclododecapeptide, valinomycin in vesicles formed from two synthetic lipids is studied by differential scanning calorimetry, spin-label partitioning electron paramagnetic resonance and [ $^1\text{H}$ ]-nuclear magnetic resonance. The results show that valinomycin is located near the head group region of dipalmitoyl phosphatidyl choline vesicles and in the hydrophobic core of the dimyristoyl phosphatidyl choline vesicles in the liquid crystalline phase.

**Keywords.** Valinomycin; dipalmitoylphosphatidylcholine; dimyristoylphosphatidylcholine; differential scanning calorimetry; electron spin resonance; nuclear magnetic resonance; ionophore-lipid interaction.

### Introduction

Valinomycin, a cyclic dodecapeptide, is a prototype of carriers (Ovchinnikov *et al.*, 1974) which enhance the cation permeability across membranes by a diffusion of the carrier-cation complex from one side of the membrane to the other. Such a diffusion would take place only when the membrane is in the liquid crystalline phase as is confirmed by temperature-dependent black lipid membrane (BLM) steady-state conductance measurements (Boheim *et al.* 1980). The carrier complexes with the cation at one interface to form a hydrophobic carrier-cation complex. This would be done with efficiency if the local concentration of the carrier at the interface is enhanced by a preferential location of the uncomplexed carrier at that interface.

Free valinomycin in solution adopts solvent dependent conformations (Mayers and Urry, 1972; Patel and Tonelli, 1973) whereas the conformation of its  $\text{K}^+$  complex is independent of the nature of the solvent (Davis and Tosteson, 1975). Since the membrane-water interface is similar to the polar organic solvents and the interior can be modelled by nonpolar organic solvents, it is likely that the conformation of valinomycin at the interfaces is different from that in the interior. We report in this paper our results on the location of valinomycin in dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) bilayers, both in the uni- and multi-lamellar vesicular forms, using differential scanning calorimetry (DSC), spin-

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Abbreviations used: BLM, Black lipid membrane; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; EYL, egg yolk lecithin; LBPT, lipid bilayer phase transition; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; TEMPO, 2,2,6,6-tetramethylpiperidin-1-oxyl; ULV, unilamellar vesicle.

label partitioning electron paramagnetic resonance (EPR) and [ $^1\text{H}$ ]-nuclear magnetic resonance (NMR) techniques.

### Experimental

Valinomycin, L- $\alpha$ -DMPC, L- $\alpha$ -DPPC, and  $\text{D}_2\text{O}$  are from Sigma Chemical Company, St. Louis, Missouri, USA. 2,2,6,6-Tetramethylpiperidin-1-oxyl (TEMPO) was synthesized according to standard procedures (Rozantsev, 1970). DSC thermograms were recorded on a Perkin-Elmer DSC-2C instrument calibrated with Indium and Benzene. Typically about 20  $\mu\text{L}$  (50 mg/mL) of the sample was placed in aluminum pans used for volatile samples. The thermograms were recorded with a heating rate of 10 deg/min and a range of 10 mcal/sec. The EPR spectra were recorded on a JEOL-FE3X spectrometer equipped with a variable temperature accessory. A standard flat quartz cell was used for recording the spectra. The EPR spectra were recorded at increasing temperatures in steps of 2°C. [ $^1\text{H}$ ]-NMR spectra were recorded on a Bruker WH 270 spectrometer. The field-frequency lock was obtained from the deuterium signal of the solvent  $\text{D}_2\text{O}$ .

For the DSC experiments, appropriate amounts of lipid and lipid-valinomycin mixtures were dissolved in chloroform. A thin film was formed by evaporating the chloroform by bubbling dry nitrogen gas through the solution. To the vacuum dried thin film, glass-distilled water was added and the dispersion was shaken thoroughly on a vortex mixture above the lipid bilayer phase transition temperature (LBPT) of the lipid.

Samples for the EPR studies were prepared as for DSC experiments except that, all the samples contained 0.1% of the EPR probe, TEMPO.

The [ $^1\text{H}$ ]-NMR studies were done on sonicated unilamellar vesicles (ULVs) whereas the multilamellar vesicles (MLVs) were used for the DSC and EPR experiments. The ULVs were formed by sonicating the MLVs (Bangham *et al.*, 1974) formed in  $\text{D}_2\text{O}$  as described for DSC samples. The MLV samples were sonicated on a Branson B-12 sonicator till the solution became clear. The sonicated vesicles are 26nm in diameter as checked by the lanthanide shift reagent method (Bergelson, 1978). The lipids were not degraded by sonication as verified by thin layer chromatography with 6:9:2:7:5  $\text{CHCl}_3:\text{CH}_3\text{OH}:7\text{M NH}_4\text{OH}$  as the eluent (Szoka and Papahadjopoulos, 1980).

### Results

The interaction of valinomycin with DPPC MLVs was studied by spin-label partitioning EPR (Shimshick and McConnell, 1973). The EPR spectra of TEMPO in DPPC MLVs containing various concentrations of valinomycin were recorded at different temperatures. The TEMPO parameter,  $P$  was plotted against temperature and the inflexion point was taken as the phase transition temperature.  $P$  was calculated as the ratio of intensity of the TEMPO signal in the lipid bilayer to the sum of the intensities in the lipid bilayer and water (Shimshick and McConnell, 1973). Such graphs for a few DPPC: valinomycin ratios are shown in figure 1. Free DPPC showed the pretransition at 35°C and the main transition at 43°C. The pretransition broadened initially and gradually disappeared as the valinomycin concentration increased. No

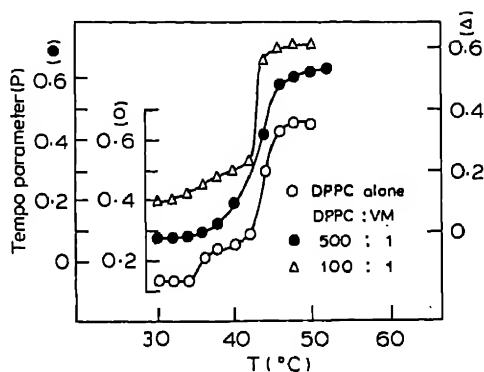


Figure 1. Phase transition temperatures of DPPC-valinomycin MLVs by spin-label partitioning EPR. [DPPC] = 0.18M, [TEMPO] = 0.75 mM.

significant changes in the melting temperatures of the main transition were noticed.

The lipid bilayer phase transition (LBPT) temperature was followed by DSC also. The thermograms for DPPC alone and with valinomycin at DPPC: valinomycin ratios of 100:1 are shown in figure 2. DPPC showed a main transition at 42°C as compared to the literature value of 41.5°C (Mabrey and Sturtevant, 1978). As in the case of the EPR experiments, the main transition did not change significantly whereas the pretransition was broadened. The DSC data for the valinomycin-DPPC MLVs have been reported

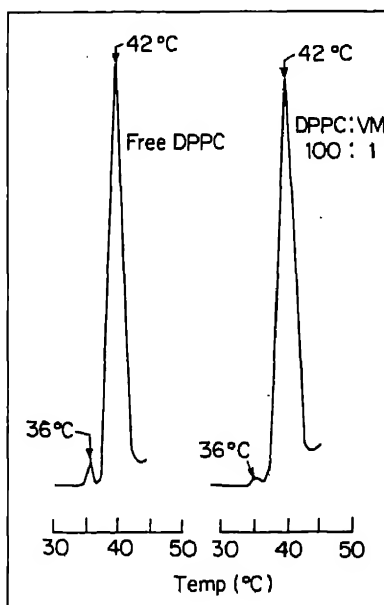


Figure 2. DSC thermograms of DPPC MLVs with valinomycin. [DPPC] = 20 mM.



earlier (Grell *et al.*, 1975) and the results showed that the main transition temperature decreased with increasing addition of valinomycin.

The  $[^1\text{H}]$ -NMR studies were carried out on ULVs from both DMPC and DPPC. In these ULVs, valinomycin was added as a methanolic solution in aliquots of 2  $\mu$ l to obtain the required lipid: valinomycin ratios. Control experiments were performed by adding the same amount of methanol to a portion of the same vesicle solution. No significant changes were observed upon addition of methanol alone.

The line widths of the choline and the aliphatic chain signals of DPPC ULVs were followed by gradual addition of valinomycin at 60°C, which is above the melting temperature. The  $[^1\text{H}]$ -NMR spectra are shown in figure 3A. The line widths of

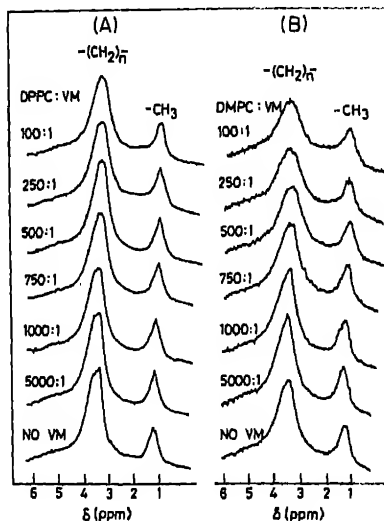


Figure 3. 270 MHz  $[^1\text{H}]$ -NMR spectra (aliphatic chain region) of (A) DPPC ULVs at 60°C and (B) DMPC ULVs at 30°C.  $[\text{DPPC}] = [\text{DMPC}] = 20 \text{ mM}$ .

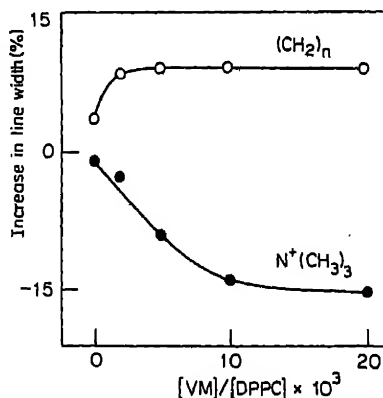


Figure 4. 270 MHz  $[^1\text{H}]$ -NMR titration graph of DPPC-valinomycin ULVs at 60°C.  $[\text{DMPC}] = 20 \text{ mM}$ .

aliphatic signals showed a slight broadening whereas the head group signals sharpened as valinomycin was gradually added. The titration graphs for the choline and the aliphatic chain signals are shown in figure 4.

The line widths of the choline and the aliphatic chain signals were followed by addition of valinomycin to DMPC ULVs at 30°C which is above its melting temperature. The  $[^1\text{H}]$ -NMR spectra are shown in figure 3B. The titration graphs are shown in figure 5. In this case, the aliphatic signals showed a significant broadening as valinomycin was gradually added and the choline signal did not show any appreciable changes.

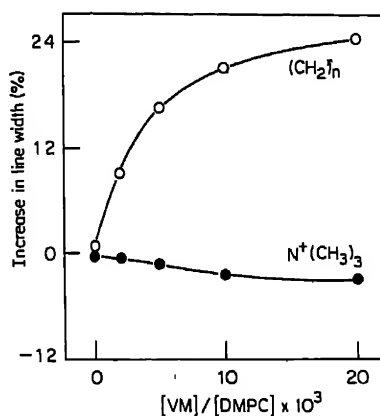


Figure 5. 270 MHz  $[^1\text{H}]$ -NMR titration graph of DMPC-valinomycin ULVs at 30°C.  $[\text{DMPC}] = 20\text{ mM}$ .

## Discussion

The  $[^1\text{H}]$ -NMR data clearly showed that valinomycin does not effectively penetrate the hydrophobic core of DPPC ULVs. The slight broadening of the methylene proton signal might be due to slower rates of chain rotations (Marsh and Watts, 1981; Chan *et al.*, 1981). The sharpening of the head group choline signals indicated a location of valinomycin near the interface. The head group has been shown to be oriented perpendicular to the bilayer normal with the negatively charged phosphate group interacting with the neighbouring positively charged choline group (Buldt and Wohlgemuth, 1981; Davis, 1983). The sharpening of the choline signal might be due to increased motion of the head group when this interaction is weakened by pulling two lipid molecules apart. A schematic representation of valinomycin in DPPC vesicles is shown in figure 6A. An earlier  $[^1\text{H}]$ -NMR study of valinomycin-DPPC MLVs has reported similar results (Hsu and Chan, 1973). Valinomycin, in this study, has been proposed to be adsorbed on the membrane surface.

The absence of any changes in the main transition as observed by DSC and EPR confirms the absence of valinomycin in the hydrophobic core of DPPC MLVs as well. The broadening of the pretransition is difficult to explain, because its origin is not well known. The pretransition has been observed in MLVs formed from phosphatidylcho-

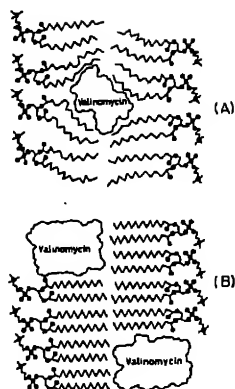


Figure 6. A. Valinomycin in DPPC vesicles. B. Valinomycin in DMPC vesicles.

lines and not from phosphatidylethanolamines (Bergelson, 1978). Based on the DSC data, the pretransition has been associated with conformational rearrangement of the head group portion of the lipid molecule (Chapman, 1975; Janiak *et al.*, 1976, 1979), while deuterium quadrupole splitting and  $[^{31}\text{P}]$ -NMR studies (Gally *et al.*, 1975) denied such conformational changes at the pretransition. Fluorescence (Jacobson and Papahadjopoulos, 1975) and NMR (McIntosh, 1980; Boroske and Trahms, 1983) studies indicated that the pretransition had a pronounced effect on the arrangement of the acyl chains. The pretransition has not been observed even in large ULVs (Takemoto *et al.*, 1981) which might not form the  $P'_\beta$  structure (Luzzati and Tardieu, 1974). Addition of certain substances like fatty acids (Mabrey and Sturtevant, 1977) and tetraphenylborate to DPPC MLVs caused a disappearance of the pretransition. The presently held opinion is that the pretransition is a consequence of some unknown effects arising from interactions among the bilayer sheets in MLVs.

In the region between the pre and the main transitions ( $P'_\beta$  phase), the chain tilt has been shown to be less than that in the gel ( $L'_\beta$  phase) and it goes to zero in the liquid crystalline phase ( $L_\alpha$ ) (Pope *et al.*, 1981). Insertion of valinomycin molecules between two lipid molecules might permit a wider range of tilt angles leading to a broadening of the pretransition. A better understanding of the origin of the pretransition is necessary to explain the effects of additives on the pretransition.

Valinomycin interacts with DMPC ULVs in an altogether different way. In this case, the aliphatic chain signals are broadened as valinomycin is added whereas the choline signals do not change significantly. This observation suggests a penetration of valinomycin into the hydrophobic core of the vesicles. The broadening of the methylene signals indicates a lipid aggregation around valinomycin which leads to motional restriction of the lipid chains. Such aggregation has been proposed for valinomycin in egg yolk lecithin vesicles (Walz, 1977, 1979). The location of valinomycin in DMPC vesicles is schematically represented in figure 6B.

Results from this study and earlier studies are summarized in table 1. These studies indicated that valinomycin is located near the head group in DPPC vesicles and in the

**Table 1.** Summary of studies on the location of valinomycin in vesicles.

Lipid	ULV/MLV	Lipid/ valinomycin	Technique	Location	Reference
DMPC	MLV	24 to 10	DSC	Interior	Grell <i>et al.</i> , (1975)
DMPC	ULV	19	CD	Interior	Grell <i>et al.</i> , (1975)
DMPC-d <sub>72</sub>	ULV	30	[ <sup>1</sup> H]-NMR	Interior	Feigenson and Meers (1980)
DMPC	ULV	1000 to 10	[ <sup>1</sup> H]-NMR	Interior	Present study
DPPC	MLV	5000 to 50	[ <sup>1</sup> H]-NMR	Head group region	Hsu and Chan (1973)
DPPC	MLV	1000 to 10	DSC, EPR	Head group region	Present study
DPPC	ULV	1000 to 10	[ <sup>1</sup> H]-NMR	Head group region	Present study
EYL	ULV	125 to 35	UV	Interior	Walz (1977, 1979)
EYL	ULV	10	CD	Interior	Grell <i>et al.</i> , (1975)

hydrophobic core in DMPC vesicles. The location is the same in both MLVs and ULVs. ULVs have been shown to be more disordered than MLVs by Raman spectroscopy (Gaber and Peticolas, 1977), fluorescence (Yguerabide and Foster, 1981) and calorimetry both above and below the melting temperature. The question of the fluidity differences between ULVs from DPPC and DMPC has not been explicitly addressed to in the literature. It appears from the available Raman spectroscopic data (Gaber and Peticolas, 1977) that DMPC vesicles are more disordered than DPPC vesicles, both in MLV and ULV forms. Valinomycin may be able to penetrate the DMPC vesicles which are more disordered than DPPC vesicles.

### Acknowledgements

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## Purification of rabbit liver phosphofructokinase and its properties under simulating *in vivo* conditions

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**Abstract.** Phosphofructokinase (EC 2.7.1.11) from rabbit liver was purified to homogeneity. Preincubation of enzyme results in nonlinearity of enzyme activity with enzyme concentration. Therefore  $K_{0.5}$  of enzyme for fructose 6 phosphate in the absence or presence of fructose 2,6 bisphosphate or polyethylene glycol or in the presence of both was determined at physiological concentrations of its various effectors by taking the initial rate obtained by adding the enzyme last. They decrease the  $K_{0.5}$  value from 4.1 mM to about 0.2 mM. The  $K_{0.5}$  of enzyme for fructose 2,6 bisphosphate was also determined under the above conditions. It is about 4.3  $\mu$ M. Transient kinetics of phosphofructokinase at varying concentrations of enzyme in the presence of fructose 2,6 bisphosphate or polyethylene glycol or in the presence of both were studied. It was found that although they decrease  $t_{1/2}$  i.e. the time to reach half the maximal steady rate by about 5–8 fold, it was about constant at varying concentrations of the enzyme. These results indicate that fructose 2,6 bisphosphate and polyethylene glycol decrease  $K_{0.5}$  of the enzyme for fructose 6 phosphate not by associating the enzyme to higher aggregates, but by a different mechanism.

**Keywords.** Phosphofructokinase; glycolysis; fructose 2, 6 bisphosphate.

### Introduction

Many reports have appeared in the last 3 years on the mechanism of regulation of liver phosphofructokinase [adenosine 5'-triphosphate (ATP): D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11] (Hers and Van Schaftingen 1982, Uyeda *et al.*, 1982; Pilkis *et al.*, 1982a). Van Schaftingen *et al.* (1981) and Uyeda *et al.* (1981) suggested that fructose 2,6 bisphosphate (F2,6P<sub>2</sub>) in liver which is most effective metabolite in activating phosphofructokinase is sufficient to keep phosphofructokinase active under *in vivo* conditions, while in its absence, Reinhart and Lardy (1980a) had shown that phosphofructokinase was hardly active at concentrations of ATP, fructose 6 phosphate (F6P) and other effectors corresponding to their *in vivo* levels in the liver at pH 7.0, and 37°C.

All the above studies on this enzyme were done by preincubating the enzyme at  $\mu$ g

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Abbreviations used: F2,6P<sub>2</sub>, Fructose 2,6 bisphosphate; F6P, fructose 6 phosphate; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetra acetic acid; F1,6P<sub>2</sub>, fructose 1,6 bisphosphate; BSA, bovine serum albumin; AMP, adenosine 5'-monophosphate; P<sub>i</sub>, inorganic phosphate; NADH, nicotinamide adenine dinucleotide adenine; ADP, adenosine 5'-diphosphate.

protein range for 2 min or 4 min (Pilkis *et al.*, 1982b) in the assay mixture, and taking the initial rate after addition of F6P, or taking the rate after the first 5 min after starting the reaction (Van Schaftingen *et al.*, 1981). No information was available whether under these conditions the enzyme activity was proportional to its concentration without which the information thus collected cannot be extrapolated to *in vivo* conditions, where the enzyme is present at many times the concentration of the enzyme in the cuvette. This information acquires importance since it was shown earlier by Tejwani (1973), that the rate of liver phosphofructokinase was not proportional to enzyme concentration at nonsaturating F6P concentration in the absence of its positive effectors, and the  $K_{0.5}$  of the enzyme for F6P through alteration of which the enzyme is basically regulated (Ramaiah, 1974) is markedly altered by preincubation of enzyme (Ramaiah and Tejwani, 1973).

Therefore, in the present paper, the enzyme from rabbit liver was purified to homogeneity, and the conditions under which the activity is proportional to enzyme concentration were determined. The enzyme activity was proportional to enzyme concentration when it is estimated in the presence of various effectors of the enzyme at their physiological concentrations. The  $K_{0.5}$  of the enzyme for F6P in the absence or presence of F2,6P<sub>2</sub>, or polyethylene glycol or in the presence of both was determined at physiological concentrations of its effectors and temperature. Reinhart (1983, 1980) suggested that F2,6P<sub>2</sub> and polyethylene glycol associate phosphofructokinase beyond tetramers, and thus decreases  $K_{0.5}$  of the enzyme for F6P. This could be verified by studying the effect of F2,6P<sub>2</sub> and polyethylene glycol on transient kinetics of phosphofructokinase. Therefore the effects of F2,6P<sub>2</sub> and polyethylene glycol on the transient kinetics of the enzyme were studied. Based on these studies, it is concluded that the phosphofructokinase activity under simulating *in vivo* concentrations of many of its effectors corresponds closely to the glycolytic rate in the normal liver, and that polyethylene glycol and F2,6P<sub>2</sub> decrease  $K_{0.5}$  of the enzyme for F6P perhaps by a common mechanism of stabilising the active form of the enzyme, rather than by associating the enzyme beyond tetramers.

## Materials and methods

### Materials

All chemicals used were of analytical grade. The biochemicals, polyethylene glycol 8000 and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Company, St. Louis, Missouri, USA.

### Rabbit livers

Rabbits were fed with laboratory diet *ad lib.* and were sacrificed by injecting air into the ear veins. Livers were quickly removed, blotted and given a quick rinse with homogenisation buffer, and then used for purification, or stored at  $-70^{\circ}\text{C}$  till further use.

### Composition of various buffers

**Homogenization buffer:** Contained 50 mM Tris-HCl, 50 mM  $\beta$ -mercaptoethanol and 5 mM Ethylenediaminetetra acetic acid (EDTA) at pH 8.0.

**Suspension buffer:** Contained the same as homogenization buffer, except EDTA and, in addition, 5 mM  $\text{MgCl}_2$ , 0.1 mM fructose 1,6 bisphosphate ( $\text{F1,6P}_2$ ) and 0.35 mM ATP.

**Dilution buffer:** Contained 50 mM Tris-HCl, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM ATP and 140 mM  $\beta$ -mercaptoethanol at pH 8.0

**Storage buffer:** Contained 50 mM Tris HCl, 0.7 M  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM  $\text{K}_2\text{HPO}_4$ , 10 mM ATP, and 0.33 mM EDTA at pH 7.5.

### Methods

Phosphofructokinase activity was determined as described earlier (Ramaiah and Tejwani, 1973). The auxiliary enzymes (200  $\mu\text{l}$  aldolase + 150  $\mu\text{l}$  triosephosphate isomerase and  $\alpha$ -glycerophosphate dehydrogenase were of Sigma Chemical Company products, No. A.6523 and G-1881 respectively) were dialyzed overnight at 0–4°C against 800 ml of 10 mM Tris-HCl buffer pH 8.0 during which time the buffer was changed once to free them of  $(\text{NH}_4)_2\text{SO}_4$ . The amount of the auxiliary enzymes that were used to assay phosphofructokinase were sufficient to ensure that the lag observed at high concentration of F6P were not due to any limitation of these enzymes. Reaction was started by addition of the enzyme. The enzyme was applied on a perspex spatula and then stirred into the assay mixture in the cuvette. The time taken for stirring and closing the cuvette chamber was about 3 to 5 sec. The specific activity of phosphofructokinase is expressed as units per mg of protein, where 1 unit is defined as the formation of 1  $\mu\text{mol}$  of  $\text{F1,6P}_2$  per min, which was computed using the molar extinction coefficient of NADH as 6270.

Lyophilized  $\text{F2,6P}_2$  kindly supplied by Van Schaftingen and Hers was estimated by hydrolyzing it to F6P which was estimated using excess commercial muscle phosphofructokinase as described by Van Schaftingen and Hers (1981).

**Protein estimation:** Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

### SDS-polyacrylamide gel electrophoresis

Phosphofructokinase and marker proteins (thyroglobulin, bovine serum albumin and ovalbumin) at final concentrations of 0.5 mg/ml were incubated for 2 h at 37°C in 50 mM sodium phosphate buffer, pH 7.1, containing 1% SDS and 1%  $\beta$ -mercaptoethanol. 25  $\mu\text{g}$  protein samples were applied on the gels along with Bromophenol Blue and a drop of glycerol. Electrophoresis in 5% gel was performed at 5 m amperes/gel (Weber and Osborn, 1969).

**Staining and destaining procedure:** At the end of the run, the gels were immersed in 5% trichloroacetic acid for 12 h and later stained with Coomassie Brilliant Blue. Destaining was done with 20% methanol and 7% acetic acid solution.



## Results

All operations, unless otherwise mentioned, were carried out at 0–4°C.

### *Purification scheme*

**Homogenization:** About 200 g of fresh or frozen livers were thawed, minced with scissors, and homogenized in two volumes of homogenization buffer in a Waring Blender for 2 × 30 sec. The homogenate was centrifuged in an SS-34 rotor at 20,000 *g* for 45 min.

**Heat-alcohol step:** 1 ml of  $\beta$ -mercaptoethanol (14.29 M) was added per 100 ml of homogenate supernatant. After mixing, 20 ml of 95% alcohol per 100 ml of the above solution was added slowly, and the solution was kept in a water bath at 41–42°C for 45 min with occasional stirring. At the end of the incubation, it was centrifuged in an SS-34 rotor at 20,000 *g* for 45 min and the clear, reddish supernatant was filtered through Whatman No. 1 filter paper. The enzyme can be stored at –70°C at this step without loss of much of its activity for at least 12 h.

**Magnesium chloride precipitation:** The filtrate from the previous step was cooled to –3°C, and 1 M  $MgCl_2$  was added slowly to a final concentration of 50 mM while the solution was constantly stirred. It was kept at –3°C for 30 min and then centrifuged in an SS-34 rotor at –3°C at 20,000 *g* for 45 min. The brown pellets obtained were pooled and suspended in 2 ml of suspension buffer per 100 g of wet weight of tissue.

**Differential centrifugation:** The suspension obtained in the preceding Step 3 was spun for 1 h in rotor Ti 55.2 at 34,000 *g*. The precipitate thus obtained had negligible activity and was discarded. The supernatant was further centrifuged at 105,000 *g* for 1 h.

**Extraction of phosphofructokinase by F6P:** The pellet obtained in step 4 was gently swirled with a solution of 2 mM F6P (0.2 ml per 100 g of original weight wet tissue) for about 20 min and the supernatant was gently pipetted out. This procedure was repeated once more and the supernatants were pooled. Most of the phosphofructokinase in the pellet was thus removed. The enzyme can be stored at this step at –70°C overnight without any loss of activity.

**Precipitation of phosphofructokinase:** The supernatant thus obtained in Step 5 was diluted four times with dilution buffer and centrifuged in rotor Ti 55.2 at 105,000 *g* for 45 min. The pellet so obtained was gently swirled with 2 mM F6P solution as described in Step 5. Steps 5 and 6 were thus repeated four times which results in the further purification of phosphofructokinase.

**$(NH_4)_2SO_4$  Precipitation:** The pellet obtained in Step 6 was suspended in storage buffer (0.2 ml per 100 g original wet weight tissue). This was diluted 6 times with a buffer containing 1.8 M  $(NH_4)_2SO_4$ , 50 mM Tris HCl, 140 mM  $\beta$ -mercaptoethanol at pH 8.0, so that the final  $(NH_4)_2SO_4$  concentration was 1.5 M. This was kept at 0°C for 30 min with occasional stirring, and then centrifuged in rotor Ti 55.2 at 20,000 *g* for 20 min. The precipitate obtained was suspended in storage buffer (0.2 ml per 100 g of wet tissue) and distributed into small tubes and stored at –70°C until used. A typical

purification data are presented in table 1. The specific activity is about 100 units/mg protein.

**Table 1.** Purification of phosphofructokinase from rabbit liver.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Recovery (%)
Homogenate supernatant	412	19809	657	0.033	1	100
Heat alcohol filtrate	335	3350	534	0.16	5	81
Magnesium chloride precipitate suspension	3.4	57.46	342	6.00	182	52
First 2 mM F6P extract	2.4	13.84	322	23.00	697	49
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate suspended in storage buffer	0.4	1.60	157	98.00	2970	24

Attempts were first made to purify rabbit liver phosphofructokinase according to the method of Massey and Deal (1973). However, it could not be purified beyond a specific activity of 6–11 by this method. Steps 1 to 3 were essentially the same as described by Massey and Deal (1973), except that ATP concentration in suspension buffer has to be 0.35 mM instead of 0.1 mM ATP for the stability of the enzyme and the 3rd step was done at  $-3^{\circ}\text{C}$  since otherwise the enzyme was inactivated.

#### *Stability of the purified enzyme*

The enzyme stored as described above, loses about 20% of its original activity within a week. However, there was no further loss of activity for at least one month of storage.

#### *Homogeneity and the size of the subunit of phosphofructokinase*

SDS gel electrophoresis of the enzyme showed a single band. The subunit molecular weight of rabbit liver phosphofructokinase determined as described by Weber and Osborn (1969) using ovalbumin, thyroglobulin, and bovine serum albumin (BSA) as standards, was found to be 80,000 (data not shown).

#### *Physiological conditions for measuring phosphofructokinase activity*

In view of the enzyme concentration dependent changes on  $K_{0.5}$  value of the enzyme for F6P (Ramaiah and Tejawani, 1973), one should study the properties of the enzyme at 50  $\mu\text{g}$  protein enzyme per ml, which is the estimated concentration of the enzyme in the cell (Reinhart and Lardy, 1980b), so as to apply the information directly to *in vivo* conditions. However, the rate will be too fast and cannot be measured accurately. But if the enzyme at physiological concentration and in the presence of its various effectors at physiological concentrations was added to an enzyme assay mixture and initial rates were taken, the properties of the enzyme at that enzyme concentration can be assumed to be true under *in vivo* conditions as well. The assay mixture described in table 2 was

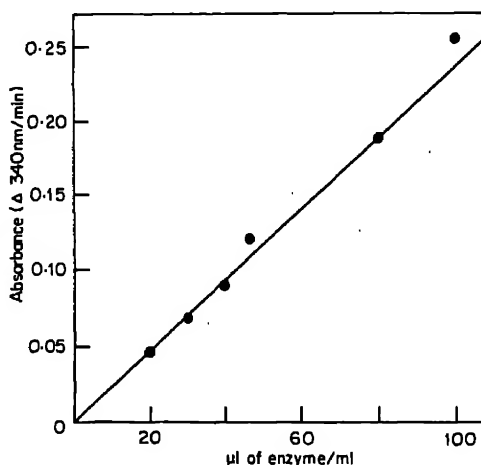
**Table 2.** Physiological concentrations of various effectors of phosphofructokinase in the liver which were used in the assay mixture.

ATP	3 mM	} Bergmeyer (1974)
Citrate	0.2 mM	
F6P	0.1 mM	
AMP	1.0 $\mu$ M	Uyeda <i>et al.</i> (1981)
$P_i$	5.0 mM	Veech <i>et al.</i> (1970)
MgCl <sub>2</sub>	5.0 mM	} Reinhart and Lardy (1980a)
KCl	120.0 mM	
Phosphofructokinase	50.0 $\mu$ g/ml	Reinhart and Lardy (1980b)

taken as close to the physiological concentrations of various effectors of phosphofructokinase except for ADP, F1,6P<sub>2</sub> and F2,6P<sub>2</sub>, which were not included in the routine assay of enzyme.

*Enzyme activity was proportional to enzyme concentration*

The enzyme from the storage buffer was first diluted to 50  $\mu$ g protein/ml of physiological assay mixture (table 2) at 0°C and to this was added 3.8  $\mu$ M F2,6P<sub>2</sub>. Various aliquots of this enzyme solution were added last to the assay mixture containing 3.8  $\mu$ M F2,6P<sub>2</sub> and the initial rate was plotted against enzyme concentration as shown in figure 1. The activity is proportional to the enzyme concentration under



**Figure 1.** Enzyme activity was assayed at 37°C with different concentrations of the enzyme in the presence of 3.8  $\mu$ M F2,6P<sub>2</sub> in physiological assay mixture which contained at final concentrations 50 mM Tris-HCl pH 7.0, 5 mM MgCl<sub>2</sub>, 5 mM  $P_i$ , 120 mM KCl, 0.2 mM citrate, 1  $\mu$ M AMP, 0.1 mM F6P, 3 mM ATP, 0.2% BSA, 0.2 mM NADH and dialyzed aldolase,  $\alpha$ -glycerophosphate dehydrogenase and triose phosphate isomerase as described in methods. The enzyme was diluted to 50  $\mu$ g protein/ml in the above physiological assay mixture excluding F6P at 0°C and was used as an enzyme source. Rate in the 1st one min after addition of enzyme was plotted against enzyme concentration.

these conditions. In the absence of F2,6P<sub>2</sub> the enzyme activity could be measured only at 5 µg protein or above/ml of physiological assay mixture. The rate was found to be proportional to the enzyme concentration.

#### *K<sub>0.5</sub> of enzyme for F6P*

The activity of enzyme at fixed concentration of enzyme was estimated at varying concentrations of F6P under conditions described in figure 1, with or without a fixed concentration of 3.8 µM F2,6P<sub>2</sub> or 25 µM F2,6P<sub>2</sub>, or 20% polyethylene glycol, and plotted as shown in figure 2. It can be seen that 20% polyethylene glycol or 3.8 µM

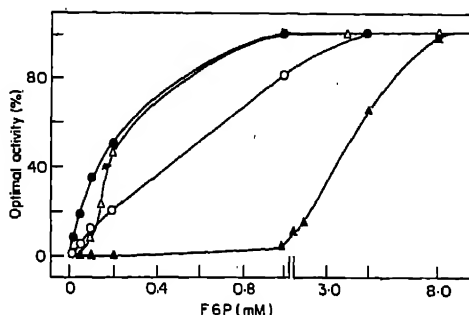


Figure 2. *K<sub>0.5</sub>* value of the enzyme for F6P in physiological assay mixture as described in figure 1 (rate in the 1st one min of enzyme activity was plotted).

(A), No F2,6P<sub>2</sub> or polyethylene glycol; (O), 3.8 µM F2,6P<sub>2</sub>; (●), 25 µM F2,6P<sub>2</sub>; (Δ), 20% polyethylene glycol (BSA was not added whenever polyethylene glycol was used in physiological assay mixture).

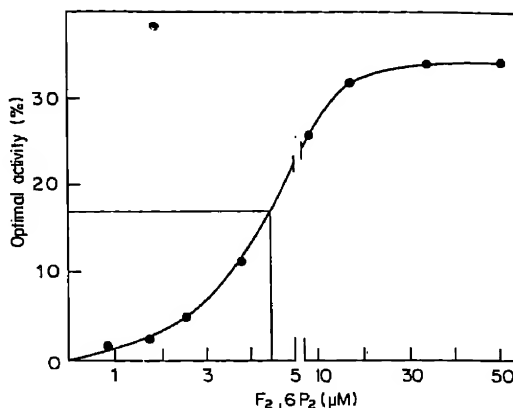
The enzyme which was diluted as described in figure 1 was used as the enzyme source in the graph (A). In other cases (O), (●), (Δ), the enzyme contained in addition 3.8 µM F2,6P<sub>2</sub> or 25 µM F2,6P<sub>2</sub> or 20% polyethylene glycol respectively.

F2,6P<sub>2</sub> or 25 µM F2,6P<sub>2</sub> lower the *K<sub>0.5</sub>* values of enzyme for F6P by about 7–18 fold. The *K<sub>0.5</sub>* value for F6P is 4.1 mM in the absence of F2,6P<sub>2</sub> or polyethylene glycol. It is reduced to 0.58 and 0.2 mM in the presence of 3.8 µM or 25 µM F2,6P<sub>2</sub> respectively. While it is 0.22 mM in the presence of 20% polyethylene glycol or in the presence of both polyethylene glycol and 25 µM F2,6P<sub>2</sub> (the data in the presence of both not shown). In the physiological range of F6P i.e. 0.1 mM F6P (Bergmeyer, 1974) and other conditions as described in figure 2 the enzyme activity is about 30% of its maximal activity showing thereby that the glycolytic rate will be quite considerable under physiological conditions in contrast to zero values expected based on the observations in figure 2 and of Reinhart and Lardy (1980a) before the discovery of F2,6P<sub>2</sub> by Van Schaftingen *et al.* (1980) and Uyeda *et al.* (1981).

#### *K<sub>0.5</sub> value of enzyme for F2,6P<sub>2</sub>*

The activity of the enzyme was measured at varying concentrations of F2,6P<sub>2</sub> under conditions described in figure 1 with a fixed concentration of F6P at 0.1 mM and the

data were plotted as described in figure 3. It can be seen that the curve is sigmoidal and the  $K_{0.5}$  is about  $4.3 \mu\text{M}$ .



**Figure 3.**  $K_{0.5}$  value of enzyme for F<sub>2,6</sub>P<sub>2</sub> in physiological assay mixture. The conditions of assay were the same as in figure 1 except that F6P was fixed at 0.1 mM and F<sub>2,6</sub>P<sub>2</sub> was varied (rate in the 1st one min of enzyme activity was taken).

#### *Acceleration of rate of reaction at high concentration of F6P*

The results described so far were done after prior dilution of enzyme to  $50 \mu\text{g}$  protein/ml and following the initial reaction after the addition of the enzyme last. The initial activity is maximum at low F6P concentrations, while at high F6P, there was acceleration of activity with time. Similar results were obtained earlier for the partially purified enzyme (Ramaiah and Tejawani, 1973) and for the pure enzyme from rat liver (Reinhart and Lardy, 1980a). In the literature many such results were explained as due to dissociation of the enzyme as a result of dilution, and that substrate induced acceleration is related to the association of the enzyme (Frieden, 1970). Recently, Reinhart (1983) suggested that F<sub>2,6</sub>P<sub>2</sub> may activate phosphofructokinase through its effect on promoting aggregation of the enzyme. But this was studied on enzyme labelled with pyrene butyrate, and assuming that polarisation values of fluorescence reflect the overall aggregation state of the enzyme (Reinhart, 1983). A study of whether such association of the enzyme was responsible for the acceleration of the enzyme activity, was done with pure enzyme preparation by measuring its activity at varying short time intervals after addition of the enzyme. The rate at any instant of time was obtained by drawing a tangent at that point on a recorder tracing of the enzyme activity curve. The data were plotted as described in figure 4. It is clear from the graph that it is a straight line indicating a first order process. Addition of F<sub>2,6</sub>P<sub>2</sub> or polyethylene glycol individually, or together decreases the  $t_{1/2}$ , i.e. the time required to reach half of the maximum steady state rate, by about 5–8 fold, and remains essentially unaltered over a five fold range of enzyme concentration as described in table 3.

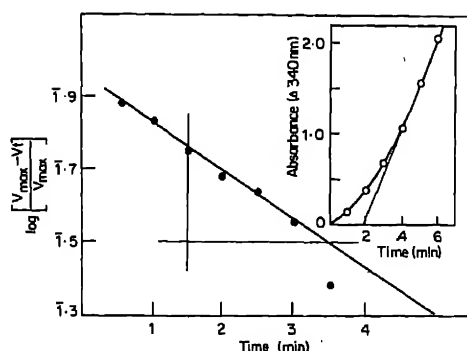


Figure 4. The enzyme was diluted as described in figure 1 and was added last to physiological assay mixture as described in figure 1 except that F6P concentration was at 5 mM. The  $V_t$  was taken from the slope of the tangent drawn from the reaction progress curve (inset) at any time  $t$ , the rate being  $\Delta A_{340} \text{ nm/min}$ .  $V_{\max}$  is the maximal steady rate under these conditions.  $\log \frac{(V_{\max} - V_t)}{V_{\max}}$  was plotted against time  $t$ .

Table 3. Enzyme assays were carried out in physiological assay mixture as described in figure 1 except that F6P was present at 5 mM and with or without 25  $\mu\text{M}$  F2,6P<sub>2</sub> or with 20% polyethylene glycol, or with 25  $\mu\text{M}$  F2,6P<sub>2</sub> and 20% polyethylene glycol together, and various amounts of the enzyme diluted as described in figure 1 were added.  $t_{1/2}$  i.e. the time required to reach half of the maximum steady state rate was calculated from reaction progress curve.

Concentration of Enzyme $\mu\text{g protein/ml}$	$t_{1/2}$ values in minutes			
	Physiological assay mixture	Physiological assay mixture with 25 $\mu\text{M}$ F2,6P <sub>2</sub>	Physiological assay mixture with 20% poly- ethylene glycol	Physiological assay mixture with 20% polyethylene glycol and 25 $\mu\text{M}$ F2,6P <sub>2</sub>
0.50	2.0	0.2	0.35	—
0.75	1.9	0.25	0.45	—
1.00	1.9	0.32	—	0.35
1.25	—	—	—	0.33
1.50	1.9	0.32	0.45	0.33
2.50	2.2	0.32	0.30	0.25

## Discussion

Reinhart and Lardy (1980a) were the first to study kinetic properties of phosphofructokinase of liver under conditions close to physiological situations, and observed that at 3 mM ATP, physiological concentrations of positive effectors [F1,6P<sub>2</sub>, AMP and inorganic phosphate ( $P_i$ )] and F6P, its activity was zero and suggested additional mechanisms to increase the enzyme affinity for F6P (1980b,c). However, this conclusion was reached before the discovery of F2,6P<sub>2</sub> by Van Schaftingen *et al.* (1980) and Uyeda *et al.* (1981) which is the most

effective activator of phosphofructokinase. Later, Van Schaftingen *et al.* (1981) and Uyeda *et al.* (1981) suggested that F2,6P<sub>2</sub> present in the liver would keep the phosphofructokinase sufficiently active, but their data cannot be extrapolated to *in vivo* situation in view of the lack of information on enzyme proportionality and due to enzyme incubation induced changes in  $K_{0.5}$  of the enzyme for F6P (Ramaiah and Tejwani, 1973). In addition estimation of F2,6P<sub>2</sub> by more sensitive method of Van Schaftingen *et al.* (1982), indicated its value to be 6  $\mu$ M in the liver of fed rat (Hue *et al.* 1983) rather than 27  $\mu$ M as estimated by Kuwajima and Uyeda (1982), or 15  $\mu$ M as mentioned by Van Schaftingen *et al.* (1981). In the present studies, it was shown that the enzyme activity was proportional to enzyme concentration under conditions described here. The  $K_{0.5}$  of the enzyme for F6P and F2,6P<sub>2</sub> determined at 37°C, and at physiological concentrations of various effectors of the enzyme, and at 3.8  $\mu$ M or 25  $\mu$ M F2,6P<sub>2</sub> were shown to be in the range of 0.58 to 0.2 mM and 4.3  $\mu$ M respectively. The glycolytic rate in the liver of fed animal is about 26% of the maximal activity of phosphofructokinase present in the liver (Reinhart and Lardy, 1980a). The activity of phosphofructokinase at 6  $\mu$ M F2,6P<sub>2</sub> is about 23% of its maximal activity (figure 3), suggesting that F2,6P<sub>2</sub>, if present at 6  $\mu$ M in the liver of fed rat, will be sufficient to explain the glycolytic rate in the liver of fed rat.

The role of phosphorylation-dephosphorylation of phosphofructokinase in its regulation is conflicting. The experiment of Sakakibara and Uyeda (1983) indicate that phosphorylated phosphofructokinase has higher  $K_{0.5}$  value for F6P and for F2,6P<sub>2</sub>, while these are contradicted by Pilkis *et al.* (1982b).

Reinhart (1980, 1983) suggested that polyethylene glycol and F2,6P<sub>2</sub> may decrease  $K_{0.5}$  of the enzyme for F6P by aggregating the enzyme to more than tetramers. But the constant  $t_{1/2}$ , i.e. the time to reach half the maximal steady rate, when rate was measured by the addition of saturating concentration of F6P in the presence of polyethylene glycol or F2,6P<sub>2</sub> or in the presence of both at varying concentrations of the enzyme indicate that perhaps no association of the enzyme was responsible for the 5–8 fold decrease in  $t_{1/2}$  in their presence as compared to the  $t_{1/2}$  in their absence (table 3). The studies on enzyme crosslinked with glutaraldehyde in the presence of its activators or at inhibitory concentration of ATP indicate also that association of enzyme may not be responsible for the acceleration of activity when the reaction was started by the addition of enzyme at high concentration of ATP and F6P (G. Roman Reddy and A. Ramaiah, unpublished results). Polyethylene glycol may not bind to the enzyme unlike that of F2,6P<sub>2</sub>, but yet may favour the active conformer of enzyme with low  $K_{0.5}$  for F6P. Polyethylene glycol may do so by preventing the enzyme to a great extent to exist in the form with high  $K_{0.5}$  for F6P, and thus favour the other form to exist to a larger extent. The observations of Lee *et al.* (1981) on lysozymes led them to suggest that polyethylene glycol may help to maintain certain conformations of proteins under conditions in which proteins might otherwise assume other structures.

### Acknowledgements

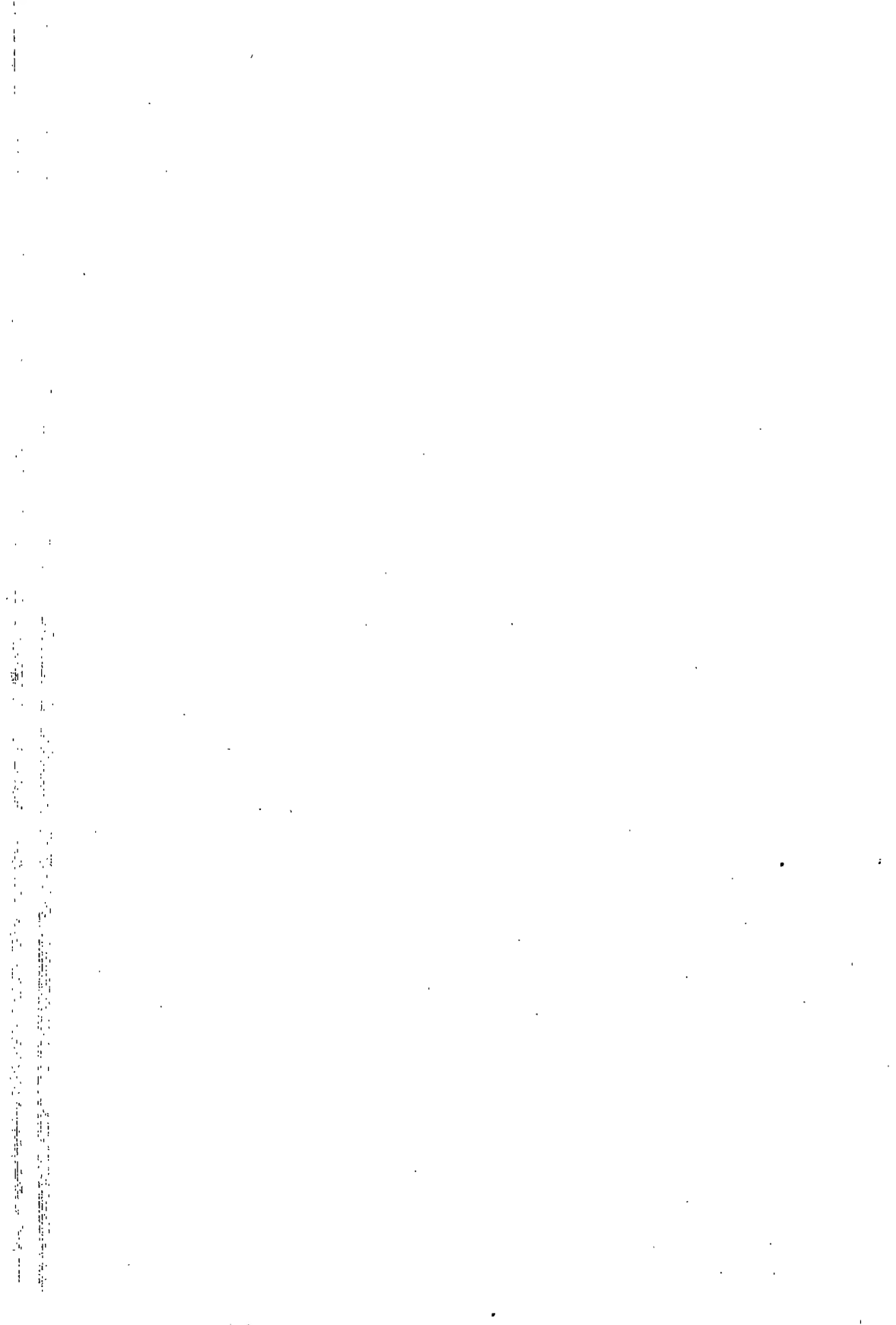
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## Relationship between relative protein value and some *in vitro* indices of protein quality

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**Abstract.** Besides amino acid composition of a protein, their bioavailability is an important determinant of the protein quality. In view of the observations over the last decade or two, implicating the small peptide uptake by the mammalian intestine as a major route of protein absorption, a few animal and plant proteins were subjected to sequential enzymatic digestion *in vitro* with pepsin, pancreatin + trypsin and erepsin and the release of amino acids as small (including dipeptides) and large peptides and free amino acids, was determined. The relative protein values of  $\alpha$ -lactalbumin, egg whites, casein, gluten, zein and protein isolates of soyabeans and groundnuts was determined using rat growth method. It was observed that relative protein value were positively correlated with the essential amino acid index of protein, quantity of essential amino acids released as small peptides and the dipeptide content of enzymatic digests, while there was a negative correlation between relative protein value and essential amino acid content of large peptide fraction.

**Keywords.** Protein quality; relative protein value; *in vitro* indices; small peptides; dipeptide release.

### Introduction

It is well recognized that amino acid (AA) composition of a protein [at least of the essential amino acids (EAAs)] determines the protein quality. However, AA composition by itself cannot predict the quality fully, because of variations in their bioavailability. Bioavailability of AAs can be influenced by several factors like protein conformation, intra and intermolecular bondings (Fukushima, 1968; Shotton and Hartley, 1970; Green *et al.*, 1973), modification of the AAs as well as the matrix of the protein in the food-stuff such as the presence of inhibitors, toxic factors, its containment in indigestible cell wall etc. (Kakade *et al.*, 1973; Pope *et al.*, 1975; Ramachandra *et al.*, 1977; Marable and Sanzone, 1981). It has been demonstrated that mammalian intestine can take up small peptides in addition to free AAs. Several kinetic advantages associated with the small peptide absorption as against the free AA absorption (Adibi, 1971; Matthews *et al.*, 1969), make the quality and quantity of digestion products of a protein, more relevant to the bioavailability of protein AAs.

Modern concepts of protein synthesis require that all AAs be available at the same time. This is determined by the rate of release of AAs from proteins during their

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Abbreviations used: AA, Amino acid; EAA, essential AA; EAAI, EAA index; PDR, pepsin digest residue; PPDD, pepsin pancreatin digest dialysate; PER, protein efficiency ratio; NPU, net protein utilization; NPR, net protein retention; SPI, soya protein isolate; GNPI, groundnut protein isolate, AN, amino nitrogen.

digestion *in vivo* by enzymes of the gastro intestinal tract. The measurement of release of at least essential amino acids (EAAs) during protein digestion *in vivo* or *in vitro* should therefore be a reasonable approach to determine the bioavailability of the AAs and hence the protein quality (Stahman and Woldegiorgis, 1975). *In vivo* studies in experimental animals (Chen *et al.*, 1962) as well as in humans (Chung *et al.*, 1976) have shown that following a protein meal, the contents of the mammalian intestine are rich in small peptides of 2-6 AA residues.

Methods based on enzymatic digestion of protein *in vitro* have long been in use to determine AA bioavailability and to predict protein quality (Melnick *et al.*, 1946). The pepsin digest residue (PDR) AA index of Sheffner *et al.* (1956), pepsin pancreatin digest dialysate (PPDD) index of Mauron (1973), Ford and Salter's (1966) enzyme digestion on gel filtration column and the multienzyme (four proteolytic enzymes) digestion method (Satterlee *et al.*, 1979) for estimating a digestibility factor to determine the quality of protein are a few of the well known *in vitro* indices of protein quality. Various *in vitro* indices have been shown to be well correlated with the quality of protein as assessed by the bioassay methods in rat or human, indicating their usefulness in predicting the quality of proteins (Sheffner *et al.*, 1967).

Several methods are available to evaluate the quality of a protein using rat growth response, such as protein efficiency ratio (PER), net protein utilization (NPU), net protein retention (NPR), relative protein value (RPV) etc. However it has been shown by Samonds and Hegsted (1976), in a collaborative study between eight laboratories, that, RPVs (Hegsted *et al.*, 1968) unlike PER or NPR were more consistent with no significant interlaboratory variations.

In view of the scanty information available on the relevance of the release of AAs as peptides during digestion of proteins, to the protein quality, an attempt was made to correlate the RPVs of a few dietary proteins with the quantity and quality of peptides released during their digestion *in vitro*, sequentially with the enzymes of the mammalian gastro intestinal tract.

## Materials and methods

Bovine serum albumin, hen egg white, casein (bovine milk), wheat gluten, corn zein and  $\alpha$ -lactalbumin (bovine milk) were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Soya protein isolate (SPI) and groundnut protein isolate (GNPI) were gifted by the Central Food Technological Research Institute, Mysore. Protein isolates of rice and redgram dhal were prepared by isoelectric precipitation, washed with acetone and dried.

Pepsin (hog stomach mucosa), trypsin (porcine) and pancreatin (hog pancreas) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA, while erepsin (hog duodenum) was obtained from Nutrition Biochemical Co., Cleveland, Ohio, USA. All other chemicals used, of analytical grade were obtained locally.

### Relative protein value determination

RPVs of seven proteins viz. casein,  $\alpha$ -lactalbumin, egg white, gluten, zein, SPI and GNPI were determined. After the protein content ( $N \times 6.25$ ) of the protein sources was

**Table 1.** Protein content of isolates and diets and the relative protein values of some protein isolates.

Protein source	Protein* (%)	Protein in diet* (%)	Slope†	RPV
Egg white	74.9	3.99, 5.66, 9.88	4.41	110.5
$\alpha$ -Lactalbumin	75.4	3.88, 6.18, 9.12	3.99	100.0
Casein	81.5	4.43, 7.56, 10.26	2.70	67.6
Soya protein isolate	88.2	8.62, 11.84, 14.79	2.26	56.7
Groundnut protein isolate	87.7	8.17, 12.93, 15.38	1.68	42.0
Wheat gluten	78.8	8.40, 12.90, 17.40	0.71	17.7
Corn zein	92.9	9.70, 13.72, 18.06	0.022	0.55

\* Protein % =  $N \times 6.25$  (as determined by Kjeldahl's method).

† Slope of the regression line between protein intake (G) and body weight gain (G).

determined by the micro kjeldahl method, three diets with different protein contents were prepared using each protein source. The protein content of the sources and the diets used are given in table 1. The composition of the basal diet is given below:

*Composition of the basal diet*

Vitamin mixture USP xix (Horowitz, 1975)	1 %
Mineral mixture (NAS-NRC, 1963)	4 %
Refined groundnut oil	10 %
Starch	85 %

Choline chloride (0.2 % w/w) and vitamins A and D (vanitin) were added to the diets in appropriate quantities. The protein sources were incorporated into the basal diet at the expense of starch, to give the desired percentage of protein in the diet. The actual method used for determining the RPVs was essentially the same as described by Pellet and Young (1980).

*In vitro* digestion of proteins

Total amino nitrogen (AN) content of the protein sources and their AA composition were determined in their acid hydrolysates (6N HCl for 20–22 h at 110°C). Integrated EAAI of these proteins were calculated using the EAA content of  $\alpha$ -lactalbumin (instead of egg white) as the reference protein (because  $\alpha$ -lactalbumin was used as reference protein in RPV determination), according to the method of Oser (1951).

The proteins, suspended in 0.05 N HCl and pH adjusted to 1.8 (1 g substance in 100 ml) were digested sequentially *in vitro* with pepsin, pancreatin + trypsin and erepsin according to the method of Ford and Salter (1966). The enzymatic digests, which contained negligible amounts of TCA precipitable material, were lyophilysed, re-dissolved in minimum quantities of glass distilled water and stored frozen at  $-20^{\circ}\text{C}$  till analysed. Aliquots of the reconstituted enzymatic digests were then loaded on columns of copper Sephadex G-25 and eluted according to the method of Fazakarley and Best (1965). The peptide and free AA fractions were freed of copper by treatment with  $\text{H}_2\text{S}$

and their AA compositions were determined in an automatic AA analyser. The percentage of each EAA of the protein, in different fractions was calculated and their average was determined. The dipeptide content of the peptide fractions was determined by a method developed for this purpose (Raghunath and Narasinga Rao 1983).

Simple correlation analyses were done between RPV of proteins, integrated EAAI of proteins, dipeptide content of the enzymatic digests, EAA content of large peptide fraction ( $P_1$ ) and EAA content of small peptide fraction ( $P_2$ ). Multiple correlation coefficient was determined between RPV, EAAI and dipeptide content of enzymatic digest, in an attempt to see, whether or not the addition of the *in vitro* digestion parameter *viz.* dipeptide content increases the correlation between RPV and EAAI. Differences between plant and animal proteins in the *in vitro* digestion indices were tested by students 't' test and modified 't' test.

## Results and discussion

Relative protein values of the proteins studied are given in table 2. The values obtained in this study are in general, in good agreement with the reported values of RPV of these proteins (Staples *et al.*, 1979; Cossack and Weber 1983). The ranking of the proteins according to the observed RPV values and the integrated EAAI tallied well and these in turn, corresponded well with the rankings reported in literature based on other quality parameters such as PER, relative growth index etc. (Jewell *et al.* 1980).

**Table 2.** Integrated essential amino acid indices, relative protein values and some *in vitro* digestion indices of some proteins.

Protein	RPV	Integrated‡ EAAI	Dipeptide† AN in enzyme digest	EAA in large* peptide ( $P_1$ ) fraction	EAA in small* peptide ( $P_2$ ) fraction
<i>Animal proteins</i>					
Bovine serum albumin	N.D.	79.05	25.9	15.1	27.1
Egg whites	110.5	114.30	46.7	—	—
$\alpha$ -Lactalbumin	100.0	100.00	34.0	15.5	25.2
Casein	67.6	78.97	28.9	23.2	36.8
Mean $\pm$ SEM			33.88 $\pm$ 4.590 <sup>a</sup>	17.9 $\pm$ 2.64 <sup>b</sup>	29.7 $\pm$ 3.59 <sup>a</sup>
<i>Plant proteins</i>					
Soya protein isolate	56.7	37.40	17.3	—	—
Groundnut protein isolate	42.0	27.40	25.9	—	—
Wheat gluten	17.7	31.30	20.1	—	—
Corn zein	0.55	30.60	15.5	41.2	8.5
Rice protein isolate	N.D.	81.40	17.9	30.7	10.4
Redgram protein isolate	N.D.	51.93	14.1	34.2	7.8
Mean $\pm$ SEM			18.47 $\pm$ 1.708	35.37 $\pm$ 3.087	8.9 $\pm$ 0.777

\* Values given are: percentage of EAAs of the protein, released into the fraction under consideration.

† Values given are: percentage of AN in the acid hydrolysate of enzymatic digest, contributed by dipeptides.

‡ Integrated EAAI determined according to the method described in the text.

Differences between animal and plant proteins: <sup>a</sup>  $P < 0.01$  by modified 't' test; <sup>b</sup>  $P < 0.02$  by students 't' test.

N.D. Not determined. —, Peptides were not separated into two fractions on copper-sephadex G 25 column.

Significant positive correlations were observed between RPV, integrated EAAI of proteins and the dipeptide content of the *in vitro* enzymatic digests of these proteins (table 3). These observations are in keeping with the well known concept that EAA content of the protein is a major determinant of the quality of the protein (Block and Mitchell, 1946). There was a significant positive correlation between EAAI and the quantity of dipeptides in the enzymatic digest indicating that EAA composition of the proteins may determine to a certain extent the type of digestion products they yield. This observation corroborates the findings of Jewell *et al.* (1980) and Bodwell (1981) who showed that content of certain AAs of protein could be used to predict its digestibility, which in turn is significantly correlated with the digestibility of the protein *in vivo* and *in vitro*.

**Table 3.** Simple and multiple correlation between *in vivo* and *in vitro* indices of protein quality of some animal and plant proteins.

Parameter	Integrated EAAI	EAA in $P_1$	EAA in $P_2$	Dipeptide in enzyme digest
RPV	0.9073 (7) <sup>a</sup>	-0.9996 (3) <sup>b</sup>	0.7351 (3)	0.8749 (7) <sup>a</sup>
Integrated EAAI	—	-0.8685 (6) <sup>b</sup>	0.6255 (6)	0.9002 (7) <sup>a</sup>

<sup>a</sup>  $P < 0.01$ ; <sup>b</sup>  $P < 0.05$ .

0.9073 and 0.8749 not significantly different from each other. Multiple correlation coefficient between RPV, integrated EAAI and dipeptide content of enzyme digest  $R = 0.9171$ .

Figures in paranthesis indicate the number of proteins used for the correlation analysis.

The multiple correlation coefficient between RPV, EAAI and dipeptide content of enzymatic digest ( $R = 0.9171$ ) was not significantly different from either the correlation between RPV and EAAI ( $R = 0.9073$ ) or between RPV and dipeptide content of enzymatic digest ( $r = 0.8749$ ). This observation indicates that addition of an *in vitro* enzyme digestibility parameter like the amount of dipeptides released during digestion may not in any significant way improve the correlation between RPV and EAA content of proteins, in agreement with the observation of Satterlee *et al.* (1977). However, there was no significant difference between the two simple correlation coefficients *viz.* those between RPV and EAAI and RPV and dipeptide content of enzymatic digest, indicating that an *in vitro* enzymatic digestion parameter such as dipeptide content of the enzyme digest could be at least as useful as the EAA composition of the protein in predicting protein quality. This finding suggests, that the quantity of dipeptides released during *in vitro* enzymatic digestion, may be useful to predict the protein quality in the same way as PDR index, PPD index, PPDD index or multienzyme digestibility index of Satterlee, etc.

In an attempt to find out whether the RPV of the protein is related to a more general *in vitro* digestion parameter like the quantity of small peptides (2.6–3.0 AA residues on an average per peptide) and large peptides (4.8–5.7 AA residues on an average per peptide) released during *in vitro* enzymatic digestion of proteins, RPV and EAAI of the

proteins were correlated with the percentage of the EAA of the protein released into large peptide ( $P_1$ ) and small peptide ( $P_2$ ) fractions. It was observed that both RPV determined experimentally and EAAI of the proteins, were negatively correlated with EAA content of large peptide fraction ( $P_1$ ) and positively correlated with EAA content of small peptide fraction ( $P_2$ ) of the enzymatic digests of proteins. These correlations, which agree well with those of Amiot *et al.* (1981), indicate that better quality proteins yield greater quantities of small peptides than poorer quality ones, while the latter class of proteins yield greater amounts of larger peptides than the former during sequential enzymatic digestion *in vitro*. Similar correlations were also observed between the EAAI and RPV of proteins and NEAA content of  $P_1$  and  $P_2$  peptide fractions (data not given here).

It was observed in this study that animal proteins which have better RPVs than the plant proteins, yield significantly greater amounts of small peptides (including dipeptides) than plant proteins which give greater quantities of large peptides (table 2). These observations which are on the expected lines, are in good agreement with the observations of Amiot *et al.* (1981) and Satterlee *et al.* (1981) who also showed that animal proteins yield higher amounts of small molecular weight peptides, while plant proteins yield more of large molecular weight peptides during their enzymatic digestion *in vitro*. In general, significantly higher quantities of all classes of amino acids (such as basic, acidic, aromatic and neutral) were released as large peptides in plant protein digests, while in case of animal protein digests though larger amounts of all classes of AAs were released as small peptides, statistical significance was observed only with basic, acidic and neutral AAs (data not given here).

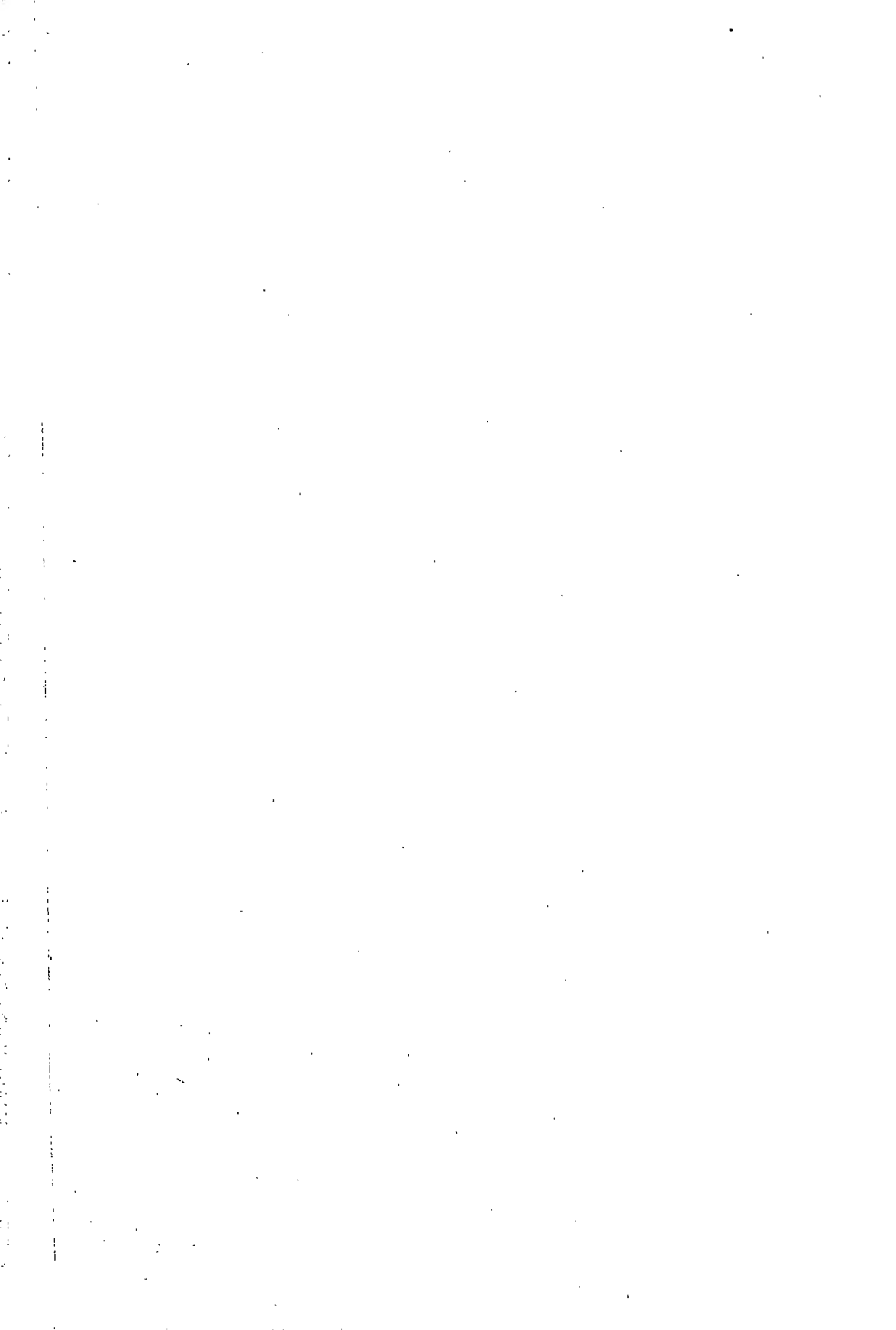
The results obtained in the present study, in general agreement with earlier such reports (Rich *et al.*, 1980; Marshall *et al.*, 1979), would thus suggest that an *in vitro* digestion parameter like the release of dipeptides during enzymatic digestion of proteins may be as useful as the EAA index of the protein itself, in predicting protein quality. It also indicates that differences do exist between plant and animal proteins in the type of enzymatic digestion products and that quantity of small peptides released, more so the dipeptides, may be an important contributory factor to protein quality.

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## An overview

The inclusion of a few papers on some of the infectious diseases in this number of the journal is intended to highlight some of the problems that have attracted the attention of the biochemists, microbiologists and immunologists in this important area.

The objective of this introductory note, is to highlight some of the areas that would need much great attention from scientists working in various disciplines in this country to answer several questions on infectious diseases, that are prevalent in our environment, and to focus on the information conveyed in the following articles.

In most bacterial, viral or parasitic infectious diseases, host-parasite interactions lead to total destruction of the host cell and multiplication of the pathogen. Alternatively tolerance of the pathogen in the host cell may permit the host cell to survive but be unable to eliminate the pathogen due to limitations in the biological/immunological potential of the host. It is also clear now that this host-parasite interaction—a manifestation of the biochemical properties of the cells is of great importance.

There are two major events that are postulated to occur when a pathogen initiates a disease state. The first one is, obtaining a spatial and biochemical foot-hold by the pathogen in any of the host cells. In most infectious diseases, these host cells could be monocytes, macrophages, epithelial cells, or tissue derived macrophages of various types. The other important step for pathogen survival is to derange the host immune mechanism. Such steps involve modification of antigen presenting cells, prevention of activation of *B/T* cells, production of suppressor *T* cells/macrophages, which release immunomodulating factors and in some instances overcoming inactivation of *T* helper cells. In many infectious diseases, while humoral immunity is unaffected, the cell mediated immunity is compromised. It is also becoming clear that cell defects lead to poor levels of important lymphokines such as Interleukin-1, Interleukin-2 and Interferon. It is being demonstrated that some of the immune defects in infectious diseases could be rectified by addition of Interleukin-2 and Interferon.

Immunotolerance is one of the major reasons for a successful survival of pathogens in human and animal hosts. This phenomenon is dramatically highlighted in a small number of humans who suffer from lepromatous leprosy. The study of the immunological aspects of leprosy, provided an opportunity to unravel some basic concepts in the immunology of infectious diseases and general immune mechanisms.

One of the more important indications has been the elucidation of a suppressor mechanism operating through a microbial component with an affinity to a group of specialised *T* cells. This component was identified as a phenolic glycolipid which appears to interact with a receptor on *T*-cells, initiating their action as suppressor cells, thus affecting both non-specific and specific antigen stimulated immune mechanisms. The suppressor mechanism has relevance to altered levels of important lymphokines, and is related to the immunotherapy by presently available "vaccines". Some of these aspects have been highlighted in the paper by Dr Bloom.

The article by Dr Brennen, highlights the role of glycolipids on the pathogenicity of leprosy bacillus, *Mycobacterium leprae*. A careful study of these compounds led to the identification, characterization and ultimately the synthesis of the phenolic glycolipid described in this article. This compound has the potential to be used as a reliable diagnostic tool for detecting leprosy and highlights the importance of biochemical investigations in this area.

The basic host-pathogen interaction during pathogenesis of the disease was demonstrated by research carried out at the Foundation for Medical Research, Bombay. One significant fact of this interaction was a change introduced in the macrophages of the host membrane by the pathogen (See Mankar *et al.* in this issue). In this study, such a basic change has been exploited to develop a screening procedure for compounds showing activity against *M. leprae*. This is another good example of practical utilisation of basic knowledge obtained from studies on this pathogenic organism.

Use of antigens and antibodies as diagnostic tools for detecting infectious diseases has been in vogue for some time and has greatly helped in the control of such diseases. A major advance in this area is the discovery of a method for immortalising antibody producing B cells, by hybridoma technique. This procedure enables the production of specific antibody with reactivity towards one antigenic epitope. In spite of a few limitations these types of antibodies have found extensive application. The paper by Reddy *et al.* illustrate how such monoclonals can be developed for use in detecting microfilaria, a widely endemic disease in this country.

The availability of gene cloning techniques, intensive studies on host-parasite interactions, application of hybridoma techniques, understanding of immune modulating mechanism and development of immuno-diagnostic tools permit a confident prediction that major infectious diseases can be conquered in the near future.

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## Immunological unresponsiveness in leprosy and its relevance to immunoregulation in man

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**Abstract.** The varied forms of leprosy form a clinical and immunological spectrum which offers extraordinary possibilities for insight into immunoregulatory mechanisms in man. At one pole, tuberculoid leprosy, patients develop high levels of cell-mediated immunity which ultimately results in killing of bacilli in the tissues, albeit often with damage to nerves. At the lepromatous pole, patients exhibit selective immunological unresponsiveness to antigens of *Mycobacterium leprae*. Even though all currently known protein species of *Mycobacterium leprae* and BCG are cross-reactive, lepromatous patients unreactive to *Mycobacterium leprae* antigens frequently respond strongly to tuberculin. *In vitro* experiments suggest the existence of lepromin-induced suppressor activity, mediated by both monocytes and  $T$  cells. The  $T$  suppressor cells have the  $T_8$  phenotype of which 50% express the activation markers,  $Ia$  and  $FcR$ . The one unique species of antigen of the leprosy bacillus is a phenolic glycolipid, and it appears that the  $T_8$  cells largely recognize the terminal trisaccharide of this unique antigen. Depletion of  $T_8$  cells restores *in vitro* reactivity of lymphocytes to lepromin in a portion of lepromatous patients, and addition of IL-2 containing supernatants partially restores responsiveness to *Mycobacterium leprae* antigens. Vaccination of lepromatous patients with a mixture of *Mycobacterium leprae* and live BCG restores cell-mediated immunity in the majority of lepromatous patients, and concomitantly reduces the *in vitro* suppressor activity and number of activated  $T_8$  cells.

These experiments suggest the existence of stage-of-disease related suppressor cells in leprosy which appear to block the responsiveness of  $T_H$  capable of responding to either specific or cross-reactive mycobacterial antigens. The mode of action of these  $T_8$  appears to be the inhibition of production of IL-2 and other lymphokines. Successful immunotherapeutic vaccination appears to overcome this block in the majority of patients.

**Keywords.** Leprosy-immunology-unresponsiveness; lepromin-induced  $T$  suppressor cells; phenolic glycolipid; interleukin-2; gamma-interferon; immunoprophylaxis.

### Introduction

From ancient times, and in virtually every culture, leprosy has evoked singular images of horror and fascination. From the immunological point of view, the disease presents a unique system for probing and intervening to control immunoregulatory mechanisms in man. As a clinical entity, leprosy presents many intriguing challenges. The etiologic agent, *Mycobacterium leprae* remains one of the very few pathogens of man that has not yet been grown in culture. There is a long latency, perhaps five years, between presumed infection and manifestation of the disease, and as a consequence the mode of

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Abbreviations used: AFB, Acid fast bacilli; ConA, concanavalin A; IL-2, Interleukin-2.

transmission remains unknown. While thirteen million people are estimated to have leprosy around the world, the disease has a relatively low prevalence, seldom exceeding 1-5/1000 in endemic areas. There is a unique fear and stigma associated with this disease, partially deriving from the deformities which occur in approximately 30 % of its victims. The deformities are most likely not a direct result of infection of nerves, but rather the consequence of immunological reactions to bacillary antigens in and around the nerves, leading to anesthesia followed by mutilation. It remains unclear why leprosy disappeared from Europe at the end of the last century, yet is currently increasing in some developing countries. Clearly, one contributory factor is the recent emergence of primary and secondary drug-resistant organisms. Although it is now known that *M. leprae* can grow in the mouse footpad, in the armadillo and most recently in the mangabey monkey, epidemiological evidence suggests that there is little or no transmission from animal to man. This offers the prospect that an effective vaccine against leprosy could, as in the case of smallpox, lead to the eradication of the disease from the face of the earth.

### *The spectrum of leprosy*

In order to describe what is known about the nature and significance of the immunological unresponsiveness in leprosy, it may be helpful to adumbrate some background information on the disease and its immunological features. Leprosy is a spectral disease that presents a diversity of clinical manifestations (Bloom and Godal, 1983; Sansonetti and Lagrange, 1981) (figure 1). A useful histopathological classification system has been developed to stage patients objectively (Ridley and Jopling, 1966) and the histopathological classification correlates extremely well with the

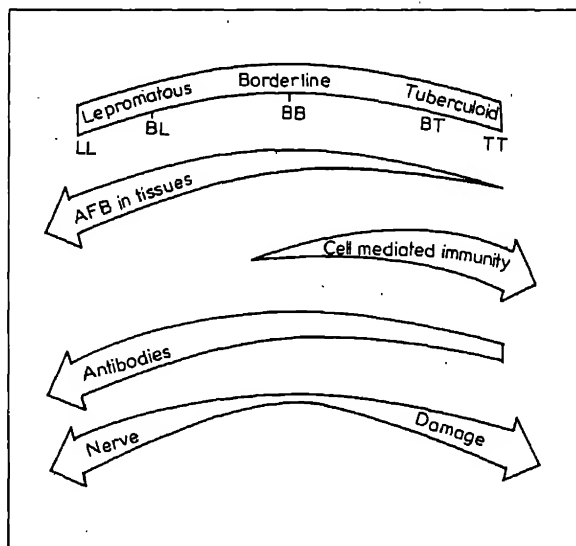


Figure 1. The spectrum of leprosy.

immunological picture. At one pole of the spectrum, tuberculoid leprosy, there are few acid fast bacilli (AFB) in the tissues, and the patients develop high levels of cell-mediated immunity, which ultimately kills and clears the bacilli in the tissues, although often with concomitant immunological damage to the nerves. At the lepromatous pole, the patients exhibit a selective unresponsiveness to antigens of *M. leprae*, and the organisms ineluctably multiply in the skin, often to extraordinary numbers, e.g.  $10^{10}$ /g tissue. Lepromatous patients have few lymphocytes in their lesions, are negative to skin testing with antigens of *M. leprae* or intact bacilli and show little or no lymphocyte transformation to *M. leprae* antigens *in vitro*. The majority of patients exist in the borderline categories between these poles, and, if untreated, gravitate towards one or the other pole of the spectrum.

Antibodies to *M. leprae* occur in all forms of the disease, but attain highest levels in lepromatous disease, suggesting that they have little to do with protection (Abe *et al.*, 1976; Young and Buchanan, 1983). Indeed, one of the major clinical problems in this form of the disease is erythema nodosum leprosum, presumed to be caused by immune complexes in the tissues (Bjorvatn *et al.*, 1976). Nerve damage occurs with increasing severity at both ends of the spectrum, contributing to the deformities found in 30% of the cases.

While leprosy is commonly described in textbooks as the least infectious disease, in fact, studies in Ethiopia (Godal, 1974) revealed that most lepromin skin test negative individuals working with active leprosy patients converted to skin test positivity in a period of several months, although they showed no evidence of disease. This implies that infection is much more common than generally assumed, and that most infections remain subclinical and are eliminated by an appropriate cellular immune response.

### *Antigens of M. leprae*

The fundamental advance that made it possible to characterize the antigens of the leprosy bacillus and to contemplate vaccines was the observation that *M. leprae* derived from lepromatous patients would grow in enormously high levels in the spleens and livers of armadillos (Kirchheimer and Storrs, 1971). It is possible to obtain  $10^{12}$  AFB from a single armadillo. Elegant purification methods were developed using percoll gradients and two-phase separation to obtain purified bacilli virtually free of contaminating host tissues (Draper, 1976). The fundamental serological finding of relevance is that when hyperimmune sera were developed against *M. leprae* and BCG and compared on crossed immunoelectrophoresis, it was observed that essentially all the protein and glycoprotein antigens detected in *M. leprae* were also recognized by antisera prepared against BCG (Harboe *et al.*, 1978). No evidence has yet emerged for a completely unique species of protein or glycoprotein in *M. leprae*. However, a number of laboratories have developed monoclonal antibodies specific for antigens of *M. leprae* (Gillis and Buchanan, 1982; Ivanyi *et al.*, 1983). The majority of monoclonal antibodies do not react with proteins on the surface of the intact organism, but rather recognize a set of *M. leprae* specific epitopes on internal polypeptides of 68 kd, 34 kd and 14 kd. It thus appears that there are unique epitopes specific for *M. leprae* found on discrete protein species which contain other determinants cross-reactive with a variety

of mycobacteria. Whether any of these epitopes is necessary or sufficient for protective immunity remains an important question.

The one unique antigen of *M. leprae* thus far identified is a complex phenolic glycolipid (Hunter *et al.*, 1982). It consists of a phthiocerol  $C_{29}$  backbone with two  $C_{30-34}$  mycocerosic acid sidechains linked by a phenolic linkage to a simple trisaccharide 3,6-di-*O*-MeGlu(1-4)-2,3-di-*O*-MeRha(1-2)-3-*O*-MeRha (*cf.* figure 9). It is this trisaccharide which is unique for *M. leprae* and contains the antigenic specificity. Monoclonal antibodies which react with the intact bacillus recognize this unique trisaccharide, as do IgM antibodies in the sera of most leprosy patients. This phenolic glycolipid-I is produced in abundance in the tissues, and only 10% of the tissue glycolipid is associated with the isolated bacilli.

### Specific unresponsiveness in lepromatous leprosy

There have been a number of hypotheses to explain the unresponsiveness of lepromatous leprosy patients to skin tests or *in vitro* lymphocyte transformation to antigens of *M. leprae*. The older view was that lepromatous leprosy patients had generalized anergy for cell-mediated immune responses. While it may be true that in untreated polar lepromatous leprosy there is depletion of the paracortical areas in lymphoid tissues and occasionally a generalized anergy, in the vast majority of patients with lepromatous leprosy there is good responsiveness to a variety of recall antigens, including reactivity to PPD. This is illustrated in figure 2, which shows the in

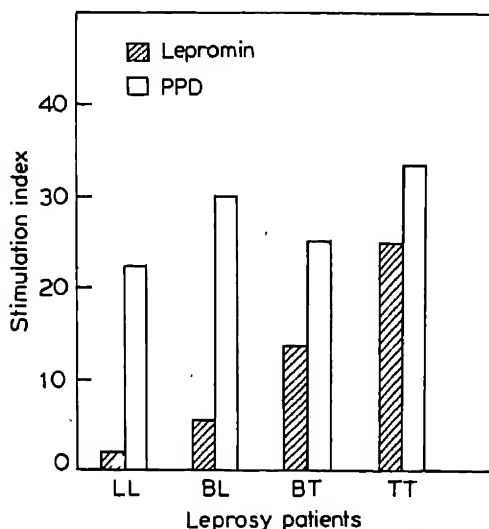


Figure 2. Stimulation of peripheral blood lymphocytes from leprosy patients by lepromin and PPD.

Peripheral blood lymphocytes were cultivated in microtiter plates at a density of  $2 \times 10^5$  cells/well in RPMI 1640 containing 10% heat-inactivated, pooled human AB serum with: (i) Dharmendra lepromin (1:10), (ii) PPD (10  $\mu$ g/ml), and [ $^3$ H]-thymidine incorporation was measured at day 6. The ratio of the mean incorporation of [ $^3$ H]-thymidine in the presence (E) or absence (C) of antigen was taken as measure of lymphocyte stimulation (stimulation index = E/C).

*vitro* lymphocyte transformation of 222 leprosy patients across the spectrum to lepromin and to PPD. It can be seen that patients throughout the spectrum show high lymphocyte transformation to tuberculin PPD, while lepromatous and borderline lepromatous patients show no or very poor lymphocyte transformation to lepromin. This selective unresponsiveness constitutes a fascinating immunological paradox. How is it possible, if most of the protein antigens of *M. leprae* are either identical or cross-reactive to those in BCG, that lymphocytes react with the antigenic determinants when they are present on the tubercle bacillus, but not when they are present on the leprosy bacillus? Currently, the most widely held view is that the unresponsiveness in lepromatous leprosy is determined by a poor constellation of immune response genes. Yet, there are several studies of segregation within families which have failed to find a significant association between HLA and lepromatous leprosy, but do reveal a weak association in segregants for tuberculoid leprosy and HLA, suggesting the possible existence of genetic 'resistance factors' linked to HLA (Fine, 1982). However, a recent study has documented an HLA association in segregation of lepromatous leprosy in 18 large families from Venezuela, indicating that there may well be some genetic factors associated with lepromatous leprosy (Van Eden *et al.*, 1983). There exists, however, a study of identical twins of which four twin pairs had discordant forms of leprosy, one with tuberculoid, the other with lepromatous, indicating that genetic factors alone are unlikely to be the principal determinant of the unresponsive state (Chakravarti and Vogel, 1973).

### Suppressor cell hypothesis

We have suggested a hypothesis that could resolve the immunological paradox in lepromatous leprosy, namely, that there must be one or a small number of unique antigenic determinants of *M. leprae* capable of engendering suppressor cells ( $T_s$ ) which have the ability to block the responsiveness of helper  $T$  cells ( $T_H$ ) to other specific or cross-reactive determinants (Bloom and Mehra, 1981). In order to try to examine such a hypothesis, and lacking HLA-matched individuals with leprosy, we developed a simple *in vitro* model for measuring the ability of *M. leprae* to induce suppression of  $T$  cell responses to the mitogen concanavalin A (ConA) (Mehra *et al.*, 1979). Lymphocytes from leprosy patients across the spectrum are stimulated with ConA alone, or ConA in the presence of lepromin, [ $^3\text{H}$ ]-thymidine incorporation is measured at 72 h, and the diminution in thymidine incorporation in the lepromin-containing culture is taken as the degree of suppression. We have found a consistent suppression of ConA stimulation in lepromatous and borderline leprosy patients, but not in tuberculoid patients, lepromin-positive contacts or normal donors (figure 3). There thus appears to be a suppressor activity specifically associated with the unresponsive form of leprosy.

### Subsets of suppressor cells

Separation of peripheral mononuclear cell population into adherent and non-adherent subsets indicated that approximately 84% of the patients had a non-adherent cell capable of suppressing the ConA responses of normal donors' lymphocytes, and about 64% had an adherent suppressor cell, presumably a macrophage, as well



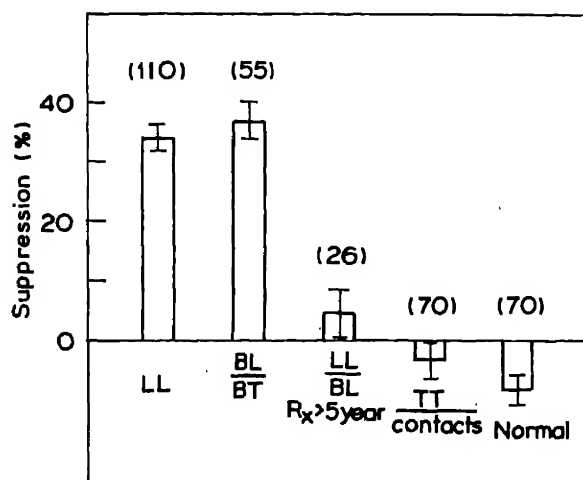


Figure 3. Lepromin-induced suppression of ConA responses of lymphocytes from leprosy patients across the spectrum.

Lymphocytes were cultured with: (i) ConA (2.5 µg/ml), (ii) ConA and Dharmendra lepromin (1:10), and (iii) no additions, as previously described (Mehra *et al.*, 1979). The cultures were labelled at 2 days with 1.0 µCi [<sup>3</sup>H]-thymidine/well and harvested 18 h later.

(table 1). Most lepromatous patients have adherent and non-adherent suppressor cells (i) but some patients have only non-adherent (ii) or only adherent (iii) *in vitro* suppressor activity. The adherent suppressor activity in lepromatous patients, presumably due to monocytes, has been confirmed and extended by studies reporting suppressive activity in extracts of lepromatous monocytes (Salgame *et al.*, 1983) and findings of a suppressor factor secreted in culture by macrophages from lepromatous

Table 1. Lepromin-induced suppression of ConA responses of normal mononuclear cells by adherent cells (monocytes) and non-adherent cells of leprosy patients.

Adherent cells	Non-adherent cells	Suppression in experiment (%)		
		I	II	III
Patient	Patient	70.6	61.2	25.0
Normal	Normal	-35.1	-24.4	2.0
—	Patient	54.1	32.9	0
Patient	Normal	41.0	-3.3	26.6
Normal	Patient	58.8	33.9	2.4

Adherent cells were separated from non-adherent cells by incubating mononuclear cell suspensions in medium containing 12.0% pooled AB serum at 37°C for 1 h, in the microtiter culture plates ( $2 \times 10^5$  cells/well). Subsequently, nonadherent cells were removed and the wells were washed twice with warm medium containing 5% FCS.  $2 \times 10^5$  nonadherent cells from normal individuals or leprosy patients were then added to each well containing autologous or allogeneic adherent cells. The cells were cultured in medium alone, ConA alone or lepromin + ConA, labelled at 2d with 1.0 µCi of [<sup>3</sup>H]-thymidine and harvested 18 h later (from Mehra *et al.*, 1979).

patients (Sathish *et al.*, 1983). A similar macrophage-like suppressor cell has been found in spleens of mice 5–10 weeks after inoculation with *Mycobacterium lepraemurium* (Bullock *et al.*, 1978).

We have pursued the characterization of the non-adherent suppressor cell in the blood of lepromatous leprosy patients (Mehra *et al.*, 1980). The non-adherent suppressor activity is totally associated with *T* cells prepared by *E*-rosetting or by cell sorting (figure 4). Using the fluorescence-activated cell sorter and monoclonal and polyclonal antibodies to lymphocyte surface markers we have shown that all of the suppressor activity in lepromatous patients is in the 20% subset recognized by OKT5 or a horse anti-human *T* cell serum (anti-*TH*<sub>2</sub>) or in the 30% subset recognized by OKT8 antibodies (figure 5), and since most studies were carried out with the latter reagent, these cells will be referred to as *T*<sub>8</sub><sup>+</sup> cells. Admixture of the *T*<sub>8</sub><sup>+</sup> subset to allogeneic normal mononuclear cells results in suppression of ConA responses of normal mononuclear cells in the presence of lepromin, indicating that the suppressor activity is not HLA restricted.

When the *T*<sub>8</sub><sup>+</sup> subset was examined for expression of *T* cell activation markers, namely *Fc* receptors and *Ia* antigens, we found that approximately 50% of the *T*<sub>8</sub><sup>+</sup> subset expressed both *Fc* receptors and monomorphic *Ia* antigens (Mehra *et al.*, 1982) (table 2). As will be documented below, monitoring of the activation markers on the *T*<sub>8</sub><sup>+</sup> subset may provide an *in vitro* means for estimating the degree of activation of these cells for suppression *in vivo*.

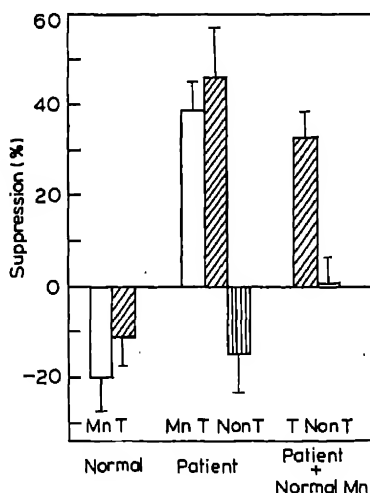


Figure 4. Suppression of ConA responses of normal mononuclear cells by *T* cells from lepromatous patients.

*T* cells were isolated from total mononuclear cells or non-adherent cells by rosetting with AET-treated sheep erythrocytes and separating the rosetted from non-rosetting cells by flotation on Ficoll-Hypaque. Subsequently, erythrocytes were lysed by 0.83% ammonium chloride. Normal mononuclear cells and patients' *T* cells were combined in 1:1 ratio. The results represent the means of six experiments (from Mehra *et al.*, 1979).

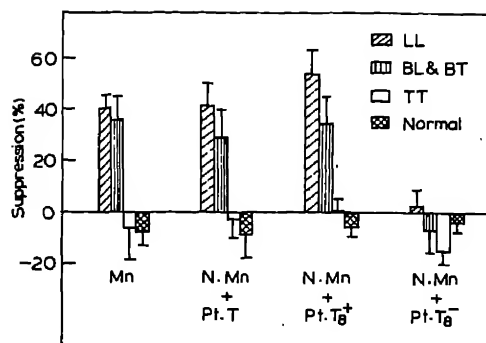


Figure 5. Lepromin-induced suppression of ConA responses of normal mononuclear cells by the  $T_8^+$  and  $T_8^-$  T cell subsets from leprosy patients.

The  $T_8^+$  and  $T_8^-$  subsets were admixed with normal mononuclear cells at a density of 25,000 sorted cells/ $1 \times 10^5$  mononuclear cells (from Mehra *et al.*, 1980).

Table 2. Expression of activation markers on T cell subsets from lepromatous patients by FACS analysis.

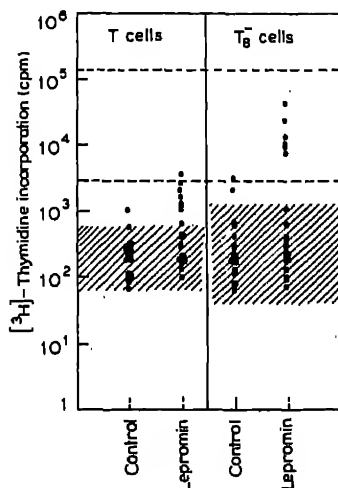
Subjects	Percentage $Ia^+$ cells among		Percentage of $FcR^+$ cells among	
	$TH_2^+/T_8^+$	$TH_2^-/T_8^-$	$TH_2^+/T_8^+$	$TH_2^-/T_8^-$
Lepromatous patients	$47.1 \pm 3.6$	$20.9 \pm 2.3$	$50.8 \pm 6.8$	$16.9 \pm 3.8$
Normals	$7.1 \pm 2.3$	$8.7 \pm 2.5$	$25.6 \pm 5.6$	$24.6 \pm 3.8$

Adapted from Mehra *et al.* (1982).

If the  $T_8^+$  subset, responsible for the lepromin-induced suppression of mitogenic responses, is related to the unresponsiveness to the antigens of *M. leprae* in these patients, then depletion of the  $T_8^+$  subset should restore responsiveness to specific antigens in a six day lymphocyte transformation in some unresponsive patients. As shown in figure 6, removal of the  $T_8^+$  cells from lepromatous lymphocytes resulted in high levels of responsiveness to lepromin at six days in about a third of the patients, exclusively patients with borderline, and not polar lepromatous leprosy. This result indicates that the unresponsiveness in at least some lepromatous patients cannot be due to the total absence of antigen responsive cells, and that some of the unresponsiveness must be attributed, particularly in BL patients, to the presence of effector suppressor cells.

#### Antigen specificity of suppressor T cells in lepromatous leprosy

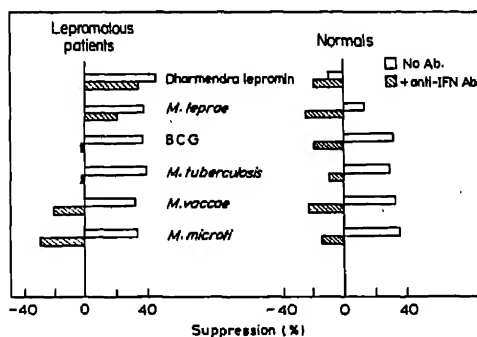
Initial studies to ascertain whether the *in vitro* suppressor activity of lepromatous lymphocytes was specific only for antigens of *M. leprae* were confounded by relatively high levels of suppression of ConA responses, even in normal individuals, induced by including any species of mycobacteria in the cultures. After some effort, we learned that all the species of mycobacteria tested induced IFN- $\alpha$  secretion in lymphocytes from



**Figure 6.** Response of peripheral blood T cells and OKT8-depleted T cells of lepromatous leprosy patients to lepromin.

Shaded area represents 95% confidence limits for the control group. Area enclosed within the broken lines indicates the 95% confidence limits for  $[^3\text{H}]$ -thymidine incorporation by peripheral blood lymphocytes from tuberculoid leprosy patients (20) stimulated with lepromin (from Mehra *et al.*, 1982).

normal, PPD-negative donors, and this correlated with the degree of non-specific suppression. Consequently, the specificity of suppression in lepromatous patients could be critically examined in the presence of a mixture of monoclonal antibodies to three species of IFN- $\alpha$  (figure 7). The results indicate that the *in vitro* suppression induced by killed mycobacteria in normal lymphocyte responses to ConA was completely eliminated by the antibodies to IFN- $\alpha$ , and that suppression seen with cultivable



**Figure 7.** Effect of monoclonal antibodies to human IFN- $\alpha$  on the suppression of ConA response induced by various mycobacteria.

Peripheral blood lymphocytes from leprosy patients and normal donors were cultured with: (i) ConA, (ii) mycobacteria ( $10 \mu\text{g/ml}$ ) + ConA, and (iii) mycobacteria, ConA and 200 units of mixture of monoclonal antibodies to three species of human IFN- $\alpha$ .

mycobacteria was similarly eliminated by the anti-IFN antibodies in lepromatous patients. In contrast, the suppression induced by Dharmendra lepromin and by purified killed *M. leprae* remained only in the lepromatous group, indicating that the  $T_S$  cells recognized only *M. leprae*-specific antigens.

Because the phenolic glycolipid-I is currently the only unique species of antigen demonstrated in *M. leprae*, in collaboration with Drs. P. Brennan and J. Convit, we examined the possibility that this phenolic glycolipid might activate the suppressor cells from lepromatous patients (Mehra *et al.*, 1984). As shown in figure 8, when glycolipid-I, which is highly insoluble in aqueous media, was incorporated in liposomes and added to lymphocytes of lepromatous patients, it was as effective as lepromin in inducing suppression. Glycolipid-I did not induce suppression in tuberculoid patients, contacts or normal donors. The optimal dose of glycolipid in liposomes inducing *in vitro* suppression was 0.5  $\mu\text{g}/\text{ml}$ . As previously, depletion of the  $T_8^+$   $T$  cell subset eliminated *in vitro* suppression observed both by lepromin and the phenolic glycolipid-I.

We were fortunate to have available a series of chemically modified *M. leprae* glycolipids and structurally related glycolipids prepared from other mycobacteria to probe the specificity of recognition of this unusual antigen by the  $T_S$  cells. As illustrated in figure 9, the results indicate that: (i) removal of the mycocerosic acid side chains (deacylated glycolipid-I) had no effect on *in vitro* suppression; (ii) removal of the terminal 3'-methyl group abolished the suppression; and (iii) removal of the terminal sugar markedly reduced the suppression. Of particular interest is the observation that no suppression was induced by the liposomes alone, or by analogous glycolipids from *M.*

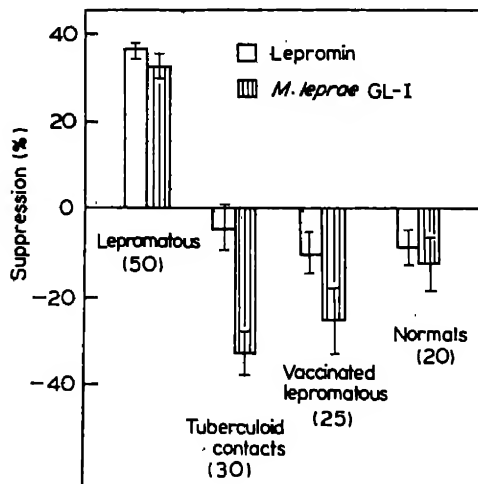
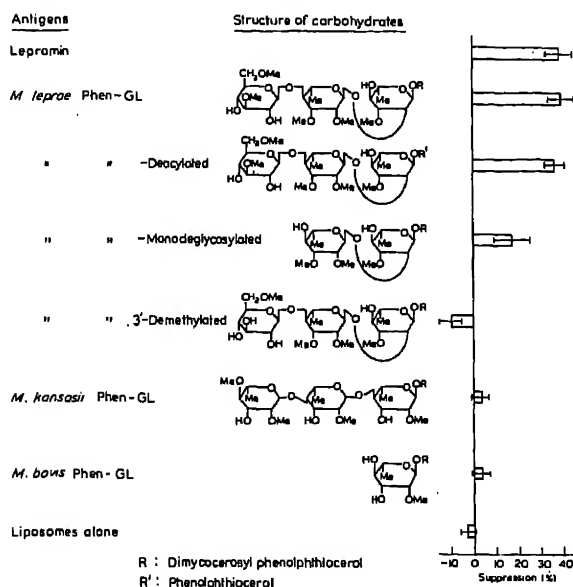


Figure 8. Suppression of mitogenic responses of peripheral blood lymphocytes of leprosy patients and normals to ConA in the presence of *M. leprae*-specific phenolic glycolipid-I and Dharmendra lepromin.

Glycolipid-I is highly insoluble in aqueous medium, and was incorporated into liposomes and then added to the lymphocytes at a concentration of 0.5  $\mu\text{g}/\text{ml}$ . Liposomes contained 2.0 mg sphingomyelin, 0.73 mg cholesterol, 0.065 mg dicetylphosphate and 0.23 mg *M. leprae* glycolipid-I in 125  $\mu\text{l}$  of Tris-NaCl buffer (pH 8.0) (from Mehra *et al.*, 1984).



**Figure 9.** Suppression of ConA responses of peripheral blood lymphocytes of lepromatous leprosy patients by modified *M. leprae* glycolipid and phenolic glycolipids from other mycobacteria.

All the preparations were added in liposomes to the cultures at 0.5 µg/ml, except deacylated GL-I, which was suspended in PBS and added at the same concentration (from Mehra *et al.*, 1984).

The chemical nature of the lipids tested are as follows:

*M. leprae* phenolic glycolipid-I:

3,6-di-O-Me-β-D-Glcp(1-4)2,3-di-O-Me-α-L-Rhap(1-2)3-O-Me-α-L-Rhap-R

*M. leprae* phenolic glycolipid-I (monodeglycosylated):

2,3-di-O-Me-α-L-Rhap(1-2)3-O-Me-α-L-Rhap-R

*M. leprae* phenolic glycolipid-III (3'-demethylated):

6-O-Me-β-D-Glcp(1-4)2,3-di-O-Me-α-L-Rhap(1-3)3-O-Me-α-L-Rhap-R

*M. kansasii* phenolic glycolipid (mycoside A):

2,4-di-O-Me-L-Rhap(1-4)2-O-Me-L-Fucp(1-4)2-O-Me-L-Rhap-R (tentative structure)

*M. bovis* phenolic glycolipid (mycoside B):

2-O-Me-α-L-Rhap-R

Glcp, glucopyranose; Rhap, rhamnopyranose, Fucp, fucopyranose; R, Dimycoerossylphenolphthiocerol

*kansasii* and *M. bovis*, which differ from *M. leprae* glycolipid-I only in the terminal saccharide moiety. These data indicate that the suppressor cell has rather exquisite specificity for the terminal trisaccharide of the phenolic glycolipid of *M. leprae*. This conclusion was confirmed by testing a series of monoclonal anti-*M. leprae* antibodies for their ability to inhibit suppression induced by lepromin or the phenolic glycolipid-I (table 3). We found that only monoclonal antibodies specific for the terminal disaccharide of the *M. leprae* glycolipid-I completely inhibited the suppression induced by glycolipid-I in liposomes and markedly reduced that by intact lepromin.

**Table 3.** Inhibition of phenolic glycolipid and lepromin induced suppression of ConA responses of peripheral blood lymphocytes of lepromatous leprosy patients by monoclonal antibodies.

Monoclonal antibodies to <i>M. leprae</i>	Specific for	Suppression of ConA response (%)	
		Phen-GL-I	Lepromin
—	—	42.9 ± 3.9	38.1 ± 2.6
46.7	Phen-GL-I terminal disaccharide	-18.9 ± 7.2	17.8 ± 6.6
$\gamma_1$	Specific epitope on 68 Kd protein	45.4 ± 3.8	27.4 ± 3.5
46.7 + $\gamma_1$	—	-23.0 ± 6.7	16.6 ± 12.5
D <sub>17</sub>	Cross-reactive with surface antigen on <i>M. leprae</i> and other mycobacteria	35.3 ± 4.9	41.8 ± 5.4
J <sub>12</sub>	Dharmendra lepromin	45.5 ± 4.2	47.9 ± 5.3

Monoclonal antibodies were produced by the fusion of spleen cells from Balb/C mice immunized with *M. leprae* to myeloma  $\times$  63 Ag 8-653 cells. The reactivity and specificity of monoclonal antibodies, all of which are IgG<sub>1</sub>, was determined by enzyme-linked immunosorbent assay analysis of 18 mycobacterial species (manuscript in preparation). In enzyme-linked immunosorbent assay, mAb 46.7 showed an O.D. of 2.23 with gly-I, 0.19 with gly-I lacking the terminal sugar, and 0.01 with gly-I lacking the terminal disaccharide.

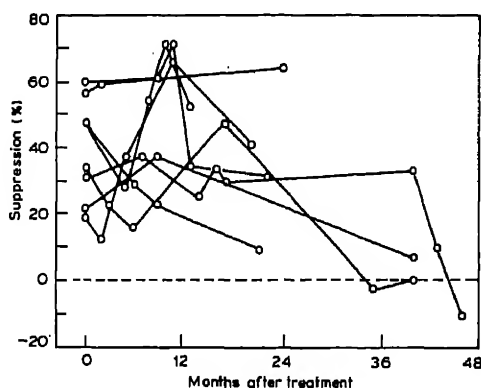
Suppression was measured as before in the presence or absence of 20  $\mu$ l/well culture supernatants from the hybrids making the monoclonal antibodies with the above mentioned reactivities (from Mehra *et al.*, 1984).

### Relationship of $T_S$ to the disease state in leprosy

Leprosy is basically a localized disease in which the battle between the immune system and *M. leprae* is fought out in the tissues, primarily skin and nerves. If the  $T_S$  cell found *in vitro* in lepromatous patients were involved in suppressing delayed-type hypersensitivity, the expansion of  $T_H$  cells and activation of macrophages, one might expect to find a predominance of  $T_8$  cells, a paucity of  $T_4$  cells, and macrophages laden with acid fast bacilli in lesions of polar lepromatous leprosy. Indeed, this is found to be the case. Using immunocytochemistry with monoclonal reagents against lymphocyte subset markers, two groups have shown that  $T_8$  cells predominate in lepromatous lesions and that  $T_4$  cells predominate in tuberculoid lesions (Van Voorhis *et al.*, 1982; Modlin *et al.*, 1983). In lepromatous lesions the macrophages are either laden with AFB or with large lipid vacuoles (the Virchow cell), recently shown to consist of the phenolic glycolipid-I (R. Modlin, personal communication). In tuberculoid lesions, there are small granulomas consisting of  $T_4$  lymphocytes and macrophages with very few AFB.

We would presume that the spectrum represents varying contributions of  $T_H$  and  $T_S$  cells within the local tissue foci of infection. Indeed, one of the extraordinary forms of the borderline disease, formerly termed dimorphic, is characterized by the presence of lesions with the histopathological characteristics of lepromatous and tuberculoid lesions simultaneously at different skin sites in the same individual. While clearcut examples of this are relatively uncommon, they serve as paradigms for the delicate regulatory balance within the lesions that determines the outcome and ultimate course of the disease.

One of the characteristics of lepromatous patients, even following elimination of most or all detectable acid fast bacilli by means of chemotherapy, is that they remain lepromin negative and unresponsive to antigens of *M. leprae* generally for the lifetime. In studies on a small number of patients with lepromatous leprosy undergoing combined chemotherapy, it was surprising to note how long lepromin-induced suppressor cells were detectable in peripheral blood, often waning only after two years of treatment (figure 10), under conditions in which killing of the vast majority of organisms was completed in 1–3 months. Of interest in this regard are observations on two polar lepromatous patients treated with dapsone, a cytostatic drug, for 10–15 years after all AFB had been cleared from the skin (Pearson, 1979). Upon cessation of chemotherapy, lesions emerged which initially took the form of borderline tuberculoid reactions and over a two-year period downgraded until they had the characteristics of florid polar lepromatous leprosy. These clinical observations indicate that long after chemotherapy some viable organisms persisted, and suggested that when they emerged there were competent *T* cells capable of forming granulomatous responses in the skin characteristic of tuberculoid lesions, which may eventually have been suppressed by the re-emergence of *T<sub>s</sub>* cells.



**Figure 10.** Lepromin-induced suppression of ConA responses of lepromatous leprosy patients undergoing combined chemotherapy for varying lengths of time, ranging from 1 month to 4 years. Patients were tested on several occasions during this period.

### *Vaccines and immunotherapy of leprosy*

It was noted many years ago that killed *M. leprae* inoculated into the skin of lepromatous patients persisted in acid fast staining form for considerable periods of time. However, when live BCG was inoculated together with killed *M. leprae*, a granulomatous response to the BCG was produced and all the acid fast bacilli were degraded, including the *M. leprae* (Convit *et al.*, 1974). This suggested the possibility that immunization with a mixture of live BCG plus *M. leprae* might bring about a state of both specific and non-specific reactivity to *M. leprae* antigens and might serve as a useful vaccine. Pursuing this rationale, Convit and his colleagues have vaccinated



several hundred lepromatous leprosy patients with a mixture of live BCG and killed purified *M. leprae* (Convit *et al.*, 1979; 1982). The results indicate that immunologic conversion, clearance of bacilli from the skin, histopathological upgrading towards the tuberculoid end of the spectrum and clinical improvement were found in approximately 85% of the borderline lepromatous patients, and in 65% of the polar lepromatous patients. These are rather dramatic results in patients who have been immunologically unresponsive often for long periods of time, and suggest a potentially general means for overcoming specific immunological unresponsiveness, at least to certain antigens, in man.

We had the opportunity to examine the *in vitro* suppressor activity and *T* cell phenotype of peripheral *T* cells from 10 such patients, in a blinded fashion, prior and subsequent to immunotherapy. The results, illustrated in figure 11, indicate that *in vitro* suppressor activity in all such vaccinated patients decreased to normal levels following immunotherapy, and expression of *Ia* was reduced to normal levels in 7 of 8 patients examined. These results provide objective evidence for immunological changes brought about by the vaccine, and establish a correlation between the *in vitro* *T* cell suppressor activity observed and the degree of the immunologic unresponsiveness *in vivo*.

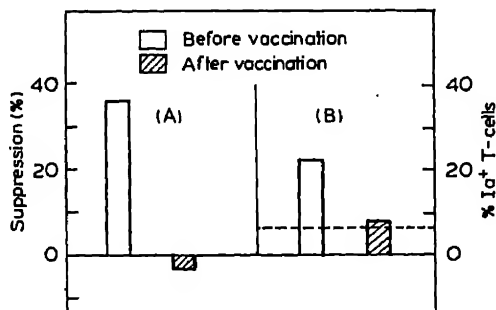


Figure 11. Diminution of *in vitro* lepromin-induced suppression of ConA responses and percentage of *Ia*<sup>+</sup> *T* cells in lepromatous patients tested both before and 18–24 months subsequent to immunotherapeutic treatment with BCG plus *M. leprae* (adapted from Mehra *et al.*, 1982).

### Relevance of findings in leprosy to general questions of immunoregulation in man

There are elaborate and sophisticated models of immunoregulatory networks in experimental animal systems (Eardley *et al.*, 1980; Greene *et al.*, 1982). For the induction of suppression in these systems, it appears that a suppressor-inducer *T* cell acts on an amplifier cell converting it to a functional suppressor cell. In general, the inducer cell has an *Lyt1*, *T<sub>H</sub>* phenotype and expresses *I-J* determinants; in some systems the amplifier cell expresses the *Lyt1*, 2,3 phenotype and the competent suppressor cell has an *Lyt2* phenotype. The first interaction is *I-J* restricted, and in some systems the effector *Lyt2 T<sub>S</sub>* cell is *I-J* unrestricted. Of interest is a recently described effector

suppressor murine *T* cell line which is carrier-specific, MHC unrestricted and expresses high levels of both *Ia* and *I-J* antigens (Nakauchi *et al.*, 1984). It would appear that the suppressor cell observed in the experiments described by us most closely corresponds to those of this *T* cell effector suppressor, in that both possess the suppressor phenotype and express high levels of *Ia*. There are several examples in man of helper-inducer cell lines, most notably in influenza in which there is a MHC-restricted suppression by *T* cells of the  $T_4$  phenotype (Fischer *et al.*, 1982). No such cell has yet been found in leprosy, although it has been reported that cultivation of lymphocytes from BCG-vaccinated normal donors with BCG results in the development of a suppressor cell *in vitro* which is MHC restricted and has a  $T_4$  phenotype (Mustafa and Godal, 1983).

While it has been possible to measure suppression of mitogen responses at 72 h, induced by lepromin, it has been very difficult to demonstrate suppression of specific responses to *M. leprae* in 6 day cultures. Attempts to suppress lepromin responses of lepromin-positive contacts using HLA-A,B, and D-matched lymphocytes from lepromatous patients have not been successful (Stoner *et al.*, 1978; Nath *et al.*, 1980). In essence, these experiments may be analogous to attempting to transfer tolerance into an already immune animal, a phenomenon that has been elicited, but only rarely and with great difficulty. This remains a problem, although it could be argued that once cells are sensitized and exposed to antigens they may be capable of producing all the lymphokines and factors required for amplification of the response *in vitro*, even in the presence of  $T_8$ , although within lesions this may be more easily regulated.

### *The role of lymphokines*

Despite the dazzling interactions of regulatory *T* cell networks in model systems, the ultimate mechanism by which suppressor cells prevent competent lymphocytes from functioning remains unresolved. Recent findings in experimental models indicate that mitogen-, alloantigen- or hapten-induced suppressor cells inhibit the proliferation of *T* lymphocytes by blocking the production of Interleukin-2 (IL-2) (Gullberg and Larsson, 1982; Kramer and Koszinowski, 1982). A soluble suppressor molecule has been obtained from *T* cells of mice rendered unresponsive to chemical allergens which mediates suppression by blocking IL-2 production by  $T_H$  cells. This factor is neither antigen-specific nor MHC-restricted (Malkovsky *et al.*, 1982).

The first evidence that a similar mechanism may pertain to suppressor cell regulation in man derives from the experiments of Haregewoin *et al.* (1983), which demonstrated that lymphocytes from lepromatous patients cultured with *M. leprae* failed to produce IL-2. More importantly, addition of supernatants from mitogen-stimulated lymphocytes known to contain IL-2 restore, at least partially, the ability of lymphocytes from lepromatous patients to respond to *M. leprae*. These results again indicate that some  $T_H$  cells reactive to *M. leprae* exist in the blood of lepromatous patients, an observation consistent with our findings that depletion of  $T_8$  cells restores *in vitro* lepromin responsiveness in a portion of these patients. This work represents the first report of some degree of restoration of immunological competence in a disease-associated state of immunological unresponsiveness in man by means of lymphokines. These studies have been extended by other studies indicating that lymphocytes from lepromatous

leprosy patients failed to produce IFN- $\gamma$  (Nogueira *et al.*, 1983), the lymphokine which appears to be required for activation of macrophages for production of  $O_2^-$  and  $H_2O_2$  (Nathan *et al.*, 1983). Exposure of the cells to IL-2 restored their ability to proliferate in response to *M. leprae* and also to produce IFN- $\gamma$ . The ability of *T* cells from lepromatous patients to respond to IL-2, and the presence of Tac receptors, the putative receptor for IL-2, on lymphocytes in lesions of leprosy patients (R. L. Modlin, personal communication) indicates that the defect in lepromatous lesions is related to the inability to produce IL-2. If so, these studies in leprosy, taken together, would provide first insight on the mode of action of  $T_S$  cells in man. It remains unclear, at present, whether there is any defect in IL-1 production in lepromatous lesions, or whether the primary defect can be pinpointed as being the failure to produce IL-2.

#### *Factors predisposing for development of $T_S$ cells in leprosy*

While there is an enormous body of literature on the requirements for *Ir* gene products and antigen presentation to  $T_H$  cells, there is very little information on the requirements for development and amplification of  $T_S$  in any system. There are very few experiments in which suppressor cells have been produced *de novo in vitro*. Interestingly, this has been reported in a mycobacterial system in mice, after cultivating *Ia*-depleted spleen cells with BCG (Nakamura *et al.*, 1982). This resulted in the development of BCG-specific  $T_S$  cells. Upon transfer of these cells in normal mice, the ability of recipients to develop delayed hypersensitivity foot pad reactions to PPD was markedly suppressed. The phenotype of the suppressor cell was *I-J* positive, and the development of suppressor cells *in vitro* could be blocked by anti-*I-J* serum.

It remains very important to identify the factors in man which determine the part of the spectrum to which any individual infected with *M. leprae* will gravitate. Clearly, the vast majority of people infected by *M. leprae* develop appropriate cell-mediated immunity and no evidence of clinical disease. It is unclear what determines whether infection will be contained or will develop towards the lepromatous or tuberculoid poles of the spectrum. One view would hold that the *Ir* genes may be determinative, although the association is sufficiently weak that, in our view, they are likely only to be contributory. Another possible explanation is the route of infection; if *M. leprae* is introduced into the skin, taken up by macrophages or presented by *Ia*-positive Langerhans cells, dendritic cells or macrophages, it is likely that some degree of cell mediated immunity will develop. If *M. leprae* is contracted by a route of infection which tends to bypass appropriate antigen presentation, possibly by the oral or respiratory routes, the possibility exists that  $T_H$  cell development will be retarded and suppressor cells will be developed by default. If this rather simplistic model of antigen presenting capability as being the determinative factor for development of different forms of disease were true, one might have expected that fewer macrophages in lepromatous lesions would express *Ia* antigens than those in tuberculoid reactions. Both studies of the histopathology of lesions have clearly found that almost all macrophages of both lepromatous and tuberculoid lesions are positive for HLA-Dr monomorphic antigens. One wonders if these determinants are sufficient for adequate presentation of complex antigens such as bacteria to  $T_H$  cells. Since the classical experiments of Macher and Chase (1969), demonstrating that the introduction of antigens simultaneously leads to the development of sensitization and unresponsiveness, it remains quite unclear, in

leprosy or any other clinical state of unresponsiveness, precisely what factors determine which form of response will develop.

#### *Mechanisms of vaccine action*

Leprosy remains the only example in man of specific immunological unresponsiveness which can, with reasonable frequency, be overcome by immunization or immunotherapy. While several potential vaccines derived from cultivable mycobacteria are being developed (Deo *et al.*, 1981; Chaudhuri *et al.*, 1983), the most extensively detailed studies on histopathology and clinical improvement are those of Convit (Convit *et al.*, 1979; 1982), vaccinating lepromatous patients with live BCG and killed *M. leprae*. It is important to try to develop an understanding in immunological terms of the mechanism by which this breaking of unresponsiveness is accomplished. Among the possibilities to be considered are: (i) BCG represents an immunologically cross-reactive antigen which can break tolerance and cause the emergence of cross-reactive clones that are protective against *M. leprae*; (ii) BCG provides an amplification of BCG-reactive clones which, in local lesions, could produce enough IL-2 to permit the expansion of that small number of *M. leprae* specific  $T_H$  remaining at a level sufficient to provide activation of macrophages and protection; (iii) BCG causes elevated expression of *Ir* gene products, perhaps as a result of local IFN production, required for antigen presentation and thereby permitting the amplification of *M. leprae*-specific  $T_H$  cells. Two approaches to the dynamics and mechanisms of immunoconversion during vaccination would be to follow the changes in lymphocyte subpopulations and production of IL-2, IL-1 and IFN- $\gamma$  in the lesions of lepromatous patients, or to treat patients with cloned IL-2 or IFN- $\gamma$  either locally or systemically.

#### *Do suppressor T cells recognize polysaccharide and lipid antigens?*

Evidence presented here suggests that the  $T_S$  cells of lepromatous patients have remarkable specificity for the terminal di- or trisaccharide of the *M. leprae*-specific phenolic glycolipid. Yet it has long been problematic whether  $T$  cells are capable of recognizing polysaccharide antigens or sugars. Earlier studies on antibody responses to pneumococcal polysaccharide III indicated a role for  $T$  suppressor cells *in vivo*, although there remains no convincing evidence for recognition of SSS-III by  $T$  cells (Baker, 1975; Braley-Mullen, 1980). Recent evidence indicates that the determinant recognized by the suppressor cell in this system is the idiotype on the IgM antibody initially formed (Taylor *et al.*, 1983). Clearly, antibody responses to hapten-coupled ficoll (Letvin *et al.*, 1981) and streptococcal A carbohydrate (Eichmann, 1975) have been found to be  $T$  cell dependent, but again there is little direct evidence that  $T$  cells recognize the carbohydrate. Delayed type hypersensitivity to tuberculo-carbohydrates has been reported (Baer and Chaparas, 1964). Recently, cell-mediated immunity to *Bacillus fragilis* has been shown to be induced by the capsular polysaccharide, and curiously, is mediated by an MHC-unrestricted,  $Lyt2, T$  cell in the mouse (Shapiro *et al.*, 1982). These findings indicate that some carbohydrate moieties can be recognized by  $T$  cells, perhaps preferentially by  $T_S$  cells.

It is not without interest that the tumor-associated antigens recognized by monoclonal antibodies in a variety of human tumor systems, including melanomas (Dippold *et al.*, 1980; Pukel *et al.*, 1982; Yeh *et al.*, 1982), Burkitt's lymphoma (Nudelman *et al.*, 1983), and colonic tumors (Koprowski *et al.*, 1981; Magnani *et al.*,

1981) have been found to be glycolipid antigens. In the case of *M. leprae*, we have been unable to demonstrate any specific *in vitro* lymphocyte transformation induced by the glycolipid-I and it may well be that this type of antigen is not readily recognized by  $T_H$  c CTL in man. What remains to be explored, however, is whether in the tumor system these immature surface glycolipids may be recognized by  $T_S$  and contribute to the immunological unresponsiveness. If  $T_S$  recognition of carbohydrate or lipid moieties is found to be a general phenomenon, since it is relatively easy to produce monoclonal antibodies to these lipid determinants, it is conceivable that anti-idiotypic antibodies could be produced which would react with the idiotype receptor on the specific  $T_S$  blocking the specific suppressor activity *in vivo*. While speculative, this could provide a novel general approach for overcoming unresponsiveness and restoring specific cell-mediated immunity to bacterial, parasitic and viral infections and tumor cells.

### Acknowledgements

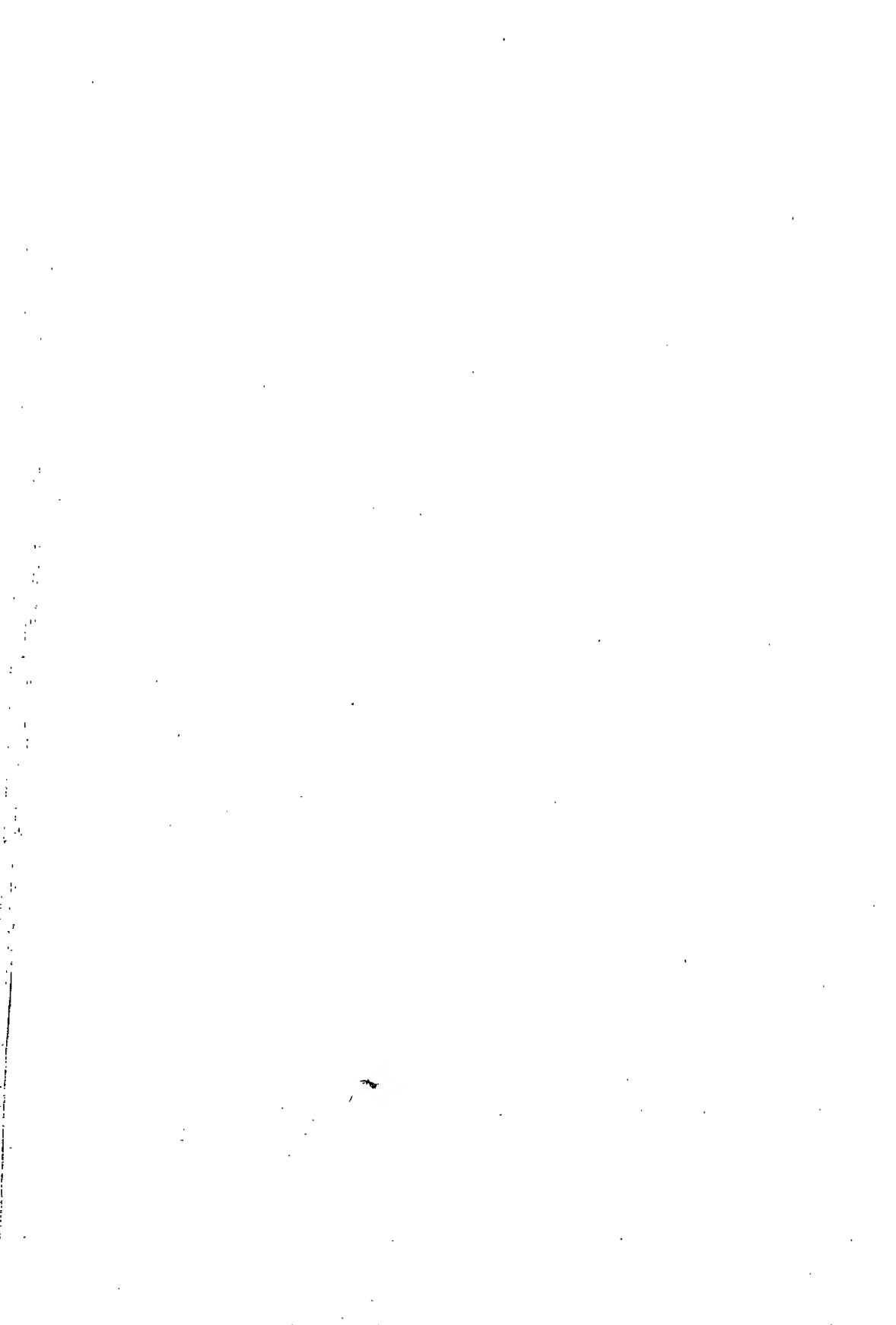
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## *Mycobacterium leprae*—The outer lipoidal surface

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**Abstract.** There is now a considerable body of evidence to suggest that the phthiocerol-containing lipids, including the phenolic glycolipids, comprise the so-called "peribacillary substance", "spherical droplets", "foamy structures" and "capsular materials" of *Mycobacterium leprae*. Thus, the phthiocerol-containing lipid capsule may be directly responsible for the intracellular survival of *Mycobacterium leprae*.

**Keywords.** *Mycobacterium leprae*; phenolic glycolipids; diacylphthiocerol; dimycocerosylphthiocerol; peribacillary nature; monoclonal antibody; enzyme linked immunosorbent assay; diagnostic tool.

The outer surface of *Mycobacterium lepraemurium* and related species such as *M. avium*, *M. scrofulaceum*, *M. intracellulare*, and *M. chelonae* have been identified chemically and characterized ultrastructurally. The subject matter has been reviewed in depth (Goren and Brennan, 1979; Draper, 1982) and succinctly (Draper, 1983; Brennan, 1983) in recent times. It is composed of a fibrillar or crystalline capsule which in the main consists of haptenic polar mycoside C glycopeptidolipids (GPL) with the general structure.

Fatty Acyl-CO-NH-D-Phe-D-a-Thr-D-Ala-L-Alaninol-O-(3,4-Me<sub>2</sub>-α-L-Rhap)



Apparently, all of the GPL-producing strains of mycobacteria are intracellular parasites and it has been suggested that the glycolipid protects bacteria from intracellular degradation (Draper and Rees, 1973). In general, the GPL-producing mycobacteria are found within phagolysosomes, and it has been proposed that GPL, due to a preponderance of D-amino acids and methoxyl substituents, is metabolically inert and accordingly provides passive protection, but which, because of its fibrillar construction, could still be permeable to nutrients (Goren, 1977; Hunter and Brennan, 1981).

*M. leprae* does not produce the kind of fibrillar capsule evident in *M. lepraemurium* and *M. avium* (Nishiura, 1960; Fukunishi *et al.*, 1982); electron microscopy has clearly established the unrelatedness between the human and murine leprosy bacillus in this respect.

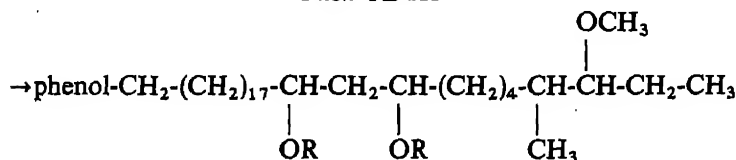
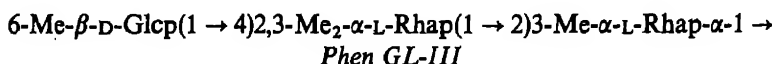
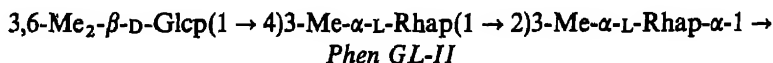
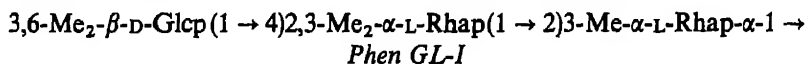
Studies of the ultrastructure of *M. leprae*, mostly *in situ* in human or armadillo tissue,



show cells about 2.5  $\mu\text{m}$  long and about 0.2  $\mu\text{m}$  wide, surrounded by a continuous cell wall composed of an inner dense layer, about 11 nm, and an extensive electron transparent outer layer (Imeada *et al.*, 1968; David *et al.*, 1978). Early investigators of the microscopic properties of *M. leprae* noted material (capsular matrices, transparent halos, sheaths) which bound the organism into clumps or globi (Hanks *et al.*, 1961). The relationship of these structures to the 8 nm diameter quasi-crystalline particles seen in sectioned *M. leprae* (David *et al.*, 1978) is not clear. However, in a recent series of elegant ultrastructural analyses, Fukunishi and others redefined the electron transparent zone and inferred that the thing and the material described in the older literature as peribacillary substance, small spherical droplets, foamy structures, and capsular materials were synonymous of substances surrounding *M. leprae* within the phagolysosomes of human leprae cells, macrophages of nude mice, or *M. leprae*-infected armadillos (Fukunishi *et al.*, 1980, 1982; Nishiura *et al.*, 1980). They further inferred that the electron transparent zones of individual bacilli coalesced with each other to form distinct intracytoplasmic foamy structures when the lesion became old, and that these were mycobacterial in origin.

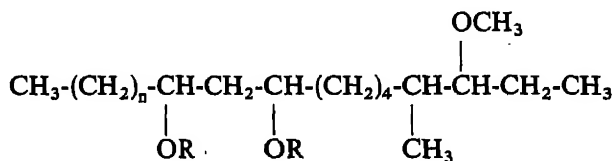
For a time it was hotly debated if these bodies were bacterial in origin. Hanks (1961), from cytological evidence and the fact that such materials were confined to the leprosy bacillus and disappeared during sulphone therapy, persuasively reasoned that they originated in *M. leprae*. Moreover, since 5–10% chloroform in aqueous systems declumped and dispersed *M. leprae*, he concluded that mycobacterial lipids were the major bonding substances in the electron transparent material. Since the material of the capsule can be stained with Sudan Black B, Fisher and Barksdale (1971) and Nishiura (1960) had concluded that the electron-transparent zone which surround *M. leprae* *in vivo* is lipid.

Members of the *Mycobacterium* genus characteristically produce lipids of extraordinary structure (Goren and Brennan, 1979; Minnikin, 1982) and *M. leprae* is no exception. Of those, the phthiocerol-containing types are the most species-specific and of the most fundamental and applied interest. To date, we have recognized and fully characterized three phenol-phthiocerol triglycosides in *M. leprae* (Hunter and Brennan, 1981, 1983; Hunter *et al.*, 1982; Fujiwara *et al.*, 1984):



R = a mixture of three mycocerosic acids; 2,4,6,8-tetramethylhexacosanoate; 2,4,6,8-tetramethyloctacosanoate; 2,4,6,8-tetramethyltriacontanoate.

In addition, the non-phenylated, non-glycosylated dimycocerosylphthiocerol has been isolated in large quantities from infected armadillo tissue and characterized completely (Hunter *et al.*, 1982; Draper *et al.*, 1983).



$$n = 16, 18$$

Phenolic glycolipid I is also present in human lepromas (Young, 1981).

Young (1981) had reported chromatographic evidence for a non-glycosylated phenol-dimycocerosylphthiocerol in *M. leprae* from a human source although as yet we have been unable to confirm this (Hunter *et al.*, 1982).

The practical implication of these glycolipids is that the trisaccharides, 3,6-di-*O*-methyl- $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  4)-2,3-di-*O*-methyl- $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  2)-3-*O*-methyl- $\alpha$ -L-rhamnopyranose in phenolic glycolipid I, and 3,6-di-*O*-methyl- $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  4)-3-*O*-methyl- $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  2)-3-*O*-methyl- $\alpha$ -L-rhamnopyranosyl in phenolic glycolipid III are highly specific for the serodiagnosis of leprosy (Cho *et al.*, 1983; Brett *et al.*, 1983; Young and Bachanan, 1983). Moreover, these oligosaccharides have been the basis of artificial antigens highly suitable for the serodiagnosis of leprosy (Fujiwara *et al.*, 1984; Cho *et al.*, 1984). For instance, coupling 3,6-di-*O*-methyl- $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  4)-2,3-di-*O*-methyl- $\alpha$ -L-rhamnopyranose to protein by reductive amination produced the product,  $\epsilon$ -N-1-[1-deoxy-2,3-di-*O*-methyl-4-*O*-(3',6'-dimethyl- $\beta$ -D-glucopyranosyl)-rhamnitol]-lysyl-bovine serum albumin which was highly sensitive in enzyme linked immunosorbant assay and showed excellent concordance with the native glycolipid in analysis of 223 sera from patients throughout the granulomatous spectrum of leprosy; the correlation coefficient, by random sampling, was 0.842.

The phthiocerol-containing lipids of *M. leprae* are extracellular and comprise the capsular electron-transparent zone. That they are extracellular was obvious from the time of their early discovery; the bulk of them were found in supernatants of homogenized leprosy-infected tissue after the bacteria had been centrifuged down (Hunter and Brennan, 1981). Further evidence that the capsular material of *M. leprae* is composed in large measure of the phthiocerol-containing lipids is indirect although persuasive. The quantities of diacylphthiocerol and phenolic glycolipid recovered from infected tissue is far in excess of that to be expected from the bacillary load, as shown by the following considerations. In recent work which is an extension and amplification of earlier work (Hunter *et al.*, 1982), we extracted 10.1 g (wet wt) of an infected armadillo liver containing  $10^{10}$  acid fast bacilli/g. The total lipid obtained was 564.8 mg. Exactly 400 mg of this total lipid fraction was injected onto a high pressure Altech silica column (25 cm  $\times$  4–6 mm, 5 micron) in  $\text{CHCl}_3$ – $\text{CH}_3\text{OH}$  (98:2) and the peak height of phenolic glycolipid I was estimated by comparing with standard preparations. In 10.1 g of the tissue there were  $1.675 \pm 0.05$  mg of phenolic glycolipid I i.e.  $165.8 \pm 5 \mu\text{g}/10^{10}$  acid fast

bacilli. This figure is 2.5 times greater than that reported previously (Hunter *et al.*, 1982). One individual cell of *M. leprae* is  $3.5 \pm 1.0 \times 10^{-14}$  g (Draper and Misell, 1977). Accordingly,  $3.7 \times 10^{10}$  acid fast bacilli amount to 1.44 mg and, in effect, according to data presented above, and previously (Hunter *et al.*, 1982), 1.44 mg of *M. leprae* produce 2 mg of cell-dissociated phthiocerol-containing lipids in the form of the diacylphthiocerol and phenolic glycolipid I.

To address directly the question of the cellular location of the phthiocerol-containing lipids, the following investigation was conducted (Hooper, L. C., Barrow, W. W., Cho, S.-N. and Brennan, P. J., unpublished results). Rabbits were inoculated with an emulsion containing 5 mg of glycolipid-methylated bovine serum albumin of which about 25% weight consisted of the glycolipid. Rabbits were bled at 3, 5 and 7 weeks postinoculation. Enzyme linked immunosorbent assay (Cho *et al.*, 1983) showed that the antiserum was directed to the phenolic glycolipid and not to any other bacterial product. The presence of *M. leprae* on tissue slides of infected armadillo tissue, prepared by pressing an impression onto glass slides (Kawamura, 1977), was then observed using the rabbit anticomplex antiserum by means of an indirect immunofluorescent antibody procedure (Barrow and Brennan, 1982). The highest dilution for which +3 to +4 fluorescence could be observed was 1:64 and peak titre was at 3 weeks postinoculation. In the infected armadillo tissue, not alone did rod-shaped bacilli fluoresce but also large patches of the surrounding tissue. The results clearly indicate that the phenolic glycolipid occupies a superficial location on the *M. leprae* cell and also appears in adjoining regions. Young *et al.* (1984) in an elegant study using anti-glycolipid monoclonal IgM have recently arrived at similar conclusions.

The anti-glycolipid rabbit antiserum and the indirect immunofluorescent procedure have also been used as an immunochemical probe to observe *M. leprae* following phagocytosis (Hooper, L. C., Barrow, W. W., Cho, S.-N. and Brennan, P. J., unpublished results). Adherent cells were allowed to phagocytose irradiated *M. leprae* and then treated with antiglycolipid antiserum followed by goat antirabbit IgG fluorescein conjugate. Examination using incident-light excitation with exciter-barrier filter and reflection combination showed that the injected bacilli had retained their glycolipid capsule.

*M. leprae* as an obligate intracellular pathogen is capable of surviving and replicating within the cells of the monocyte-macrophage system, and the mechanism by which it resists destruction by phagocytes is not at all understood. Possibilities in the context of oxidative killing are: an ability to combat phagocytic microbicidal activity by inhibiting the respiratory burst; an inability to stimulate the production of reactive oxygen metabolism; prevention of the interaction of the oxygen metabolites with the bacillus following phagocytosis; or resistance to the oxygen metabolites. A disarrangement of non-oxidative antimicrobial systems is also possible. The evidence summarized here of a capsule composed of a surfeit of chemically inert phthiocerol-containing lipid highly suited for the role of passive protectors of the resident bacterium, clearly implicate those substances in resistance to intracellular degradation. Hence, the phthiocerol-containing lipids may be regarded as virulence factors in leprosy; their chemical comportment with a considerable degree of natural methyl substitution in sugar, phthiocerol and acyl substituents alike should neutralize the attention of toxic oxidative metabolites or host degradative enzymes. A possible explanation of the vast

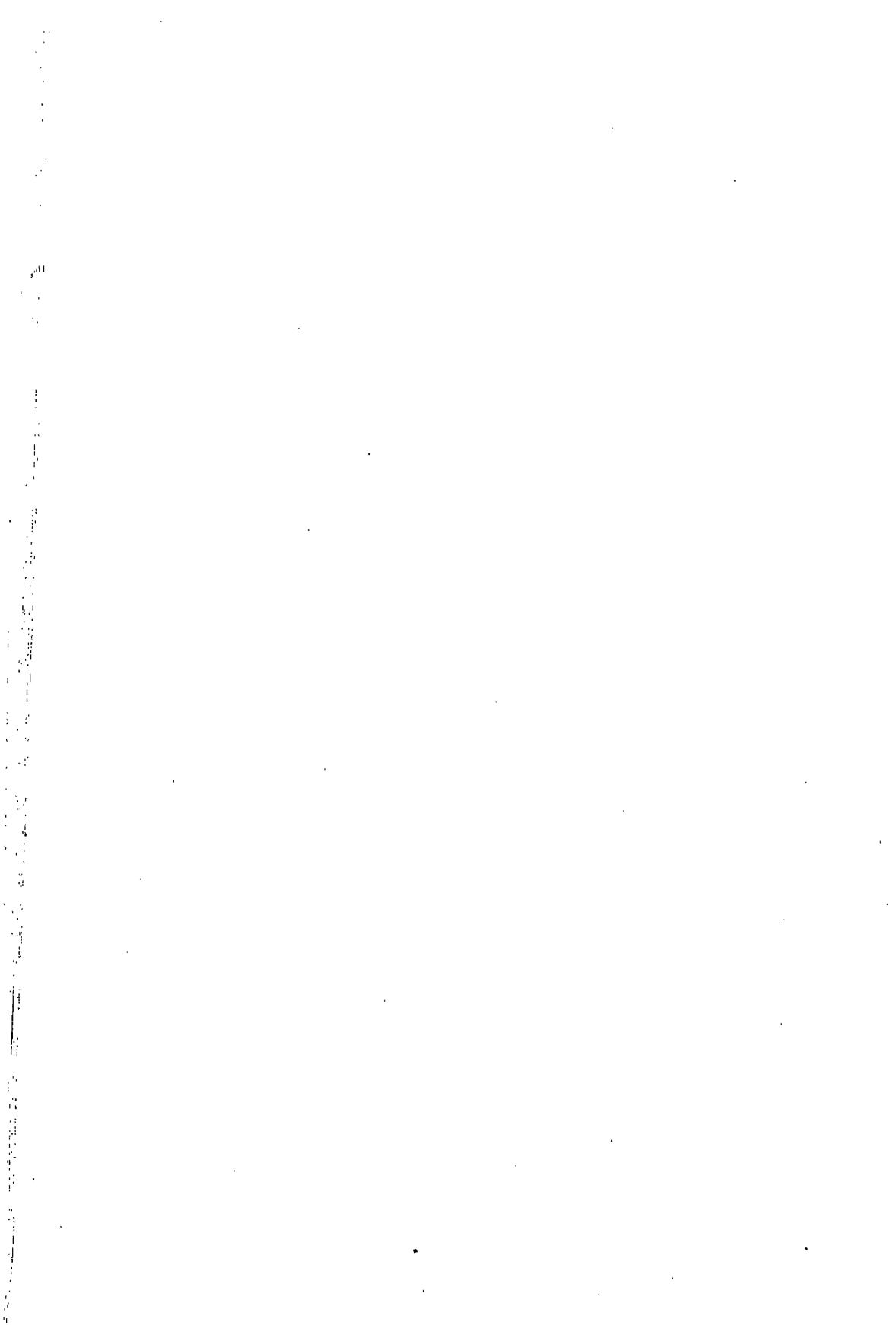
quantities of this material surrounding globi of bacilli is that they represent the skeletal remains of past bacilli, the sole survivors of lysosomal degradation of the cell wall from earlier cells which help perpetuate the remaining persistent bacilli.

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## Immunodiagnosis of bancroftian filariasis—Problems and progress

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**Abstract.** The immunodiagnosis of bancroftian filariasis is a major challenge to the immunoparasitologist. Significant progress is yet to be made in developing convenient laboratory animal model and in *in vitro* cultivation of filarial parasites making it very difficult to obtain required amount of parasite material for research. Parasitological examination techniques are not useful in low microfilaraemia, occult or chronic filarial infections. A precise and accurate immunodiagnostic technique is very much needed for successful filaria control programmes. Such a test will also avoid the need for laborious night blood examination in bancroftian filariasis.

Due to comparatively easy availability, a good amount of work has been done to explore immunodiagnostic potential of heterologous filarial antigens isolated from *Litomosoides carinii*, *Dirofilaria immitis*, *Brugia malayi*, *Setaria digitata*, *Setaria cervi* and number of other filarial species. However, there has been limited or no significant success due to number of false negative and false positive reactions.

Extensive study has also been made with antigens isolated from *Wuchereria bancrofti* microfilariae. Soluble antigens of microfilariae have been used in different immunological techniques such as skin test, counter immuno electrophoresis, indirect haemagglutination test, indirect fluorescent antibody test and enzyme linked immunosorbent assay. Fractionation of *Wuchereria bancrofti* microfilarial soluble antigens yielded mfS3e antigen fraction which was found to be highly reactive in microfilaraemia by enzyme linked immunosorbent assay, but the yield of the purified antigen was not sufficient enough to make it a practical proposition for large scale isolation of antigen.

*Wuchereria bancrofti* microfilarial excretory-secretory antigens were found to be specific and highly sensitive requiring as little as 0.35 ng antigen protein per well in penicillinase enzyme linked immunosorbent assay for detection of filarial antibody. One ml of culture fluid was found to be sufficient for 400,000 tests. Field evaluation of this test showed that it can replace laborious night blood examination.

Assay systems have been developed for detection of filarial antigen in serum, urine, hydrocele fluid and immune complexes using immunoglobulins from chronic filarial sera and antisera to excretory filarial antigens. Further purification of excretory-secretory antigens by affinity chromatography and production of monoclonal antibodies should hopefully give suitable reagents for use in sensitive assays such as enzyme immuno assay and immuno radiometric assay, providing an ideal assay system for detection of active filarial infection in the not too distant future.

**Keywords.** Immunodiagnosis; filarial antibody; filarial antigen; enzyme linked immunosorbent assay; bancroftian filariasis.

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Abbreviations used: GD, Gel diffusion; CIE, counter immuno electrophoresis; IHAT, indirect haemagglutination test; IFAT, indirect fluorescent antibody test; ELISA, enzyme linked immunosorbent assay; ES, excretory-secretory; IRMA, immunoradiometric assay, FSI, filarial serum immunoglobulin; mfS, soluble microfilarial antigen; PEG, polyethylene glycol; CIC, circulating immune complex.

## Introduction

Bancroftian filariasis is an infectious disease produced in man by the filarial parasite *Wuchereria bancrofti*. It is a major public health problem affecting about 250 million persons in tropical countries (Duke, 1978). In India alone 22 million people harbour microfilariae in blood while 16 million suffer with filarial disease manifestations such as hydrocele, lymphoedema and elephantiasis. From 25 millions in 1953, the figure rose to 304 million Indians in 1981 who are exposed to the risk of filarial infection (Sharma *et al.*, 1983). Although almost never directly fatal, chronic infection can lead to disability and disfigurement causing untold pain, misery and impairment of health in the developing world. The clinical course of lymphatic filariasis can be divided into asymptomatic, acute and chronic stages. We know so little about the parasite from the time when it disappears beneath the surface of the skin through infective mosquito bite, until some nine months later it announces its survival by the production of microfilariae. When in health and undisturbed, the parasite seems to be an expert in host's immune evasion and lives in perfect harmony with its host for considerable periods, giving a challenge to the immunologists.

One of the essential requirements for effective filaria control programme is a precise and accurate diagnostic test for detection of filarial infection on mass scale in field surveys. A definite diagnosis of filariasis is still based on the demonstration of microfilariae in peripheral blood collected at night by various parasitological examination techniques such as thick smear, counting chamber procedure, concentration test and nucleopore membrane filtration. These are not useful in low microfilaraemia, occult or chronic infection. Hence there is a need for simple, sensitive and specific immunodiagnostic test which would be of great value in nocturnally periodic *W. bancrofti* infection as a means of avoiding laborious night blood surveys and in serology for early detection.

## Immune responses

Serum immunoglobulins IgG and IgE were found to be at elevated level in bancroftian filariasis compared to controls (Subrahmanyam *et al.*, 1976). However, specific IgE antibody levels were found to be lowered in chronic filariasis when assayed by solid phase radioimmuno assay (Hussain *et al.*, 1981) and enzyme linked immunosorbent assay (ELISA) (Malhotra, *et al.*, 1984b) using *Brugia malayi* and *W. bancrofti* antigens respectively. It is interesting to note that antibodies to microfilarial surfaces (sheath) as measured by immuno fluorescent assay are usually absent in microfilaraemia but present in chronic filariasis. However on sonication cuticular and cytoplasmic antigens of microfilariae are exposed and thus antibody to these antigens could be detected in microfilaraemia sera as well (Wong and Suter, 1979; Hedge and Ridley, 1977; Kaliraj *et al.*, 1979a). Similar observation (Subrahmanyam *et al.*, 1976) was made when skin test was performed using soluble *W. bancrofti* microfilarial antigen. Antibody was found to be absent in microfilaraemia but was present in chronic filariasis. This is possibly due to the neutralization of cuticular or sheath antibodies by excess of antigen present in the circulating blood in microfilaraemia. Microfilarial surfaces were observed to acquire

blood group antigens (Ridley and Hedge, 1977) and serum albumin (Maizels *et al.*, 1984) which possibly help in parasite survival by immune evasion. In filariasis with chronic pathology, usually microfilariae are absent in peripheral blood. Sera from cases of elephantiasis promoted an intense adhesion of peripheral blood leucocytes to *W. bancrofti* microfilariae *in vitro*. The adhesion was complement independent and was associated with the IgG fraction in the human system. Antibody-dependent cellular cytotoxicity studies showed the involvement of neutrophils and macrophages as major cell types (Mehta *et al.*, 1981). In contrast cell mediated immunity was found to be suppressed in chronic filariasis as observed by leucocyte migration inhibition and lymphocyte transformation assays. Though no such suppression was observed in low microfilaraemia, however in filaria cases with high microfilarial density ( $\geq 50$  mf/c.mm), suppression of cell mediated immunity could be observed (Raghunath *et al.*, 1980; Ottesen *et al.*, 1977). Piessens (1981) rightly described lymphatic filariasis in humans as an immunologic maze.

### Immunodiagnostic tests

For an immunodiagnostic test to be acceptable as a tool for diagnostic and epidemiological purposes, it should satisfy certain requirements in sensitivity, specificity, cross reactivity, predictive value and reproducibility. Further it should be simple to perform, economic costwise, acceptable to population and should be adaptable for field study. Immunological methods such as precipitin and immunodiffusion, complement fixation, immuno and counter immuno electrophoresis, indirect haemagglutination and flocculation, immunofluorescence, ELISA, chemiluminescent ELISA radio immunoprecipitation polyethylene glycol (PEG) assay, immunoradiometric assay (IRMA), intradermal test, cell mediated immunity test, passive cutaneous anaphylaxis, *in vitro* histamine release assay and immune adherence have been explored to develop useful test for the diagnosis of different parasitic infections. The different techniques used in parasite serology and their evaluation have been published (Voller and de Savigny, 1981; Houba, 1980). Kagan (1981) classified some of these techniques based on the reactivity. Gel diffusion, complement fixation and latex agglutination are of low reactivity requiring high concentration of antibody and are not useful for detection of antibody within first week of infection. Tests of 'medium reactivity' are the indirect haemagglutination and indirect immunofluorescence techniques which can detect antibody by the second week of infection. Tests of 'high reactivity' are the radio immuno assay and ELISA. They can detect low concentrations of antibody (nano gram or pico gram/ml) on 3rd or 4th day of infection in some cases.

#### *Immunodiagnosis based on the detection of antibody in serum*

The immunodiagnosis of filariasis is one of the major challenges to the immunoparasitologist. The progress in sero diagnosis was reviewed by Kagan (1963) and Ambroise-Thomas (1980).

#### *Heterologous antigens*

The non availability of the human parasite (*W. bancrofti*) in required quantity for



antigen extraction has become an obstacle for the progress in filaria diagnosis. Hence sharing of antigens (cross reactivity) by different filarial parasites has been exploited in various immunological tests for diagnosis of filariasis. The antigens of *B. malayi* (Grove and Davis, 1978), *Setaria digitata* and *Setaria cervi* (Dissanayake and Ismail, 1981; Tandon, *et al.*, 1981). *Litomosoides carinii* (Rao *et al.*, 1980; Das gupta *et al.*, 1980), *Dirofilaria immitis* (Sawada *et al.*, 1968) and number of other species have been explored for their diagnostic potential for bancroftian filariasis. Gidel *et al.* (1969) conducted a trial of two immunological tests namely intradermal test and complement fixation test for diagnosis of filariasis using purified antigens from *D. immitis* by Sawada but without success. Studies on the detection of filarial antibody using heterologous antigens were not useful in development of a specific diagnostic test due to significant number of false positive and false negative reactions. Thus the heterologous antigens have limited or no potential use for immuno diagnosis of filarial infection.

### *Homologous antigens*

Specificity depends on the quality of the antigen employed and acceptable levels of specificity can only be obtained by using homologous and purified antigens. Though there are some encouraging leads, significant progress is yet to be made in the development of suitable animal model and in *in vitro* cultivation of human filarial parasite. Until then infected mosquitoes and humans are the only sources for *W. bancrofti* infective larvae and microfilariae respectively making it difficult to get required parasite material. Hence studies using homologous (*W. bancrofti*) antigens are scanty.

### *Somatic antigens (W. bancrofti)*

Studies have been made to explore the utility of *W. bancrofti* microfilarial antigens in gel diffusion (GD), counter immuno electrophoresis (CIE), indirect haemagglutination test (IHAT), indirect fluorescent antibody test (IFAT) and ELISA for detection of filarial antibody in filarial sera. GD was found to be least sensitive and the sensitivity in detection of antibody in maximum number of positive cases increased with each test in the above order and ELISA was observed to be highly sensitive (Kaliraj, 1980). Naidu *et al.* (1984) used *W. bancrofti* microfilarial as well as infective larval antigens in CIE and IHAT and observed that both the antigens were comparable in reactivity in IHAT. *W. bancrofti* infective larval antigen was used in skin test and positive reaction in all proven filarial infection cases as well as in endemic normals was observed (Chandra *et al.*, 1974). However when soluble microfilarial antigen was used in skin test, positive reaction in chronic filariasis and negative reaction in microfilaraemia were observed (Subrahmanyam *et al.*, 1976). Antibodies were detected against microfilariae, larvae and adult worms of *B. malayi* (Grove and Davis, 1978; Wong and Guest, 1969) and microfilariae and larvae of *W. bancrofti* (Kaliraj *et al.*, 1979a; Yong, 1973) in filarial infections by IFAT. From these studies it may be concluded that measurement of antibodies to surface antigens of adult worms is a useful indicator of infection while antibodies to surface antigens of microfilariae are correlated with disease. Using soluble *W. bancrofti* mf antigen, the efficiency of IHAT, IFAT and ELISA tests were compared for the detection of antibody in filarial sera. Filarial antibody could be detected in 93 %, 100 %, 81 % of the microfilaraemics, 75 %, 90 %, 100 % of chronic pathology and none

of the non endemic sera by IHAT, IFAT and ELISA respectively. However, 45–65% endemic normal sera showed the presence of filarial antibody by these tests. *W. bancrofti* mf antigen showed cross reaction against *Ancylostoma duodenale* sera in IHAT and ELISA when a few non endemic nonfilarial helminth infected sera were tested, necessitating further purification of this antigen to be useful for diagnosis (Kaliraj *et al.*, 1981b,c).

Soluble antigens (mfS) isolated from *W. bancrofti* microfilariae were fractionated by Sephadex G-150 gel filtration into 3 antigenic fractions (mfS1, 2 and 3). The mfS3 fraction was weakly reactive in IHAT but the same was found to be highly reactive in ELISA. The mfS2 antigen fraction showed cross reaction with non filarial helminth infected sera similar to the crude soluble antigen (mfS). The antigenic fractions (mfS1 and mfS3) were further fractionated by DEAE cellulose chromatography. Analysis by ELISA showed that mfS1b and mfS3e antigen fractions were highly active in the detection of filarial antibody in chronic filariasis (85%) and microfilaraemia (88%) sera respectively (Kaliraj *et al.*, 1982). However, processing of microfilariae from 100 ml of blood sample containing  $\geq 50$  mf/20 c.mm, gives antigen fraction (mfS3e) just sufficient for about 2000 tests, not a practical proposition for large scale isolation of antigen for field surveys.

#### Excretory-secretory antigens

*W. bancrofti* microfilarial excretory-secretory (ES) antigens were obtained by maintaining *W. bancrofti* microfilariae in medium 199 (3–4 thousand mf/ml of medium) supplemented with organic acids and sugars of Grace's medium but without serum (Kharat *et al.*, 1980). Utility of *W. bancrofti* ES antigen has been explored in IHAT (Kharat *et al.*, 1981), CNBr-Sepharose IFAT (Kharat *et al.*, 1983) and Penicillinase ELISA (Kharat *et al.*, 1982) for detection of filarial antibody. When used in ELISA, ES antigen was found to be highly sensitive and fairly specific compared to somatic antigen. As little as 0.35 ng ES antigen protein per well was found to be sufficient in detecting filarial antibody (Kharat *et al.*, 1982) compared to earlier study (Kaliraj *et al.*, 1982) with soluble microfilarial antigen (1.5  $\mu$ g/well) or fractionated antigen (0.1  $\mu$ g/well). One ml of culture fluid may be diluted to 4.0 liters and thus can be used for 400000 tests by penicillinase ELISA. Antibody isotype analysis revealed the presence of IgM antibody in all micro-filaraemics and IgG antibody in all cases with chronic pathology while some of each group contained both IgM and IgG antibodies. Reciprocal of the antibody titre varied from 1280 to 20 million in filarial sera. The level of specific antibody (IgM or IgG) titre did not show any correlation either between microfilaraemia and clinical filariasis or with microfilariae density (20–120 mf/20 c.mm) or clinical status of filaria patients (Kharat *et al.*, 1982). High ELISA antibody titre (1:1000000) was also observed in clinical toxocariasis with the culture antigen (de Savigny *et al.*, 1979). Fractionation of ES antigens by membrane filtration gave ES4 antigen fraction, which was found to be a glycoprotein in nature and was highly reactive in microfilaraemia sera (Reddy *et al.*, 1984a). *W. bancrofti* mf ES antigen specific IgE antibody was detected in filariasis and tropical eosinophilia by immunofluorescence assay (Kharat *et al.*, 1983) and ELISA (Malhotra *et al.*, 1984b). These studies showed that detection of ES antigen specific IgG + M + A antibodies

will be more useful than specific IgE for the immuno-diagnosis of filariasis. Antigen have been isolated from microfilaraemia sera, hydrocele fluid, immune complexes and urine samples of filarial patients and were found to be useful in detecting filaria antibody, providing other sources for isolation of filarial antigen of diagnostic importance (Reddy, M. V. R., Malhotra, A., Prasad, G. B. K. S. Hamilton R. G. and Harinath, B. C., unpublished observations).

### Immunodiagnosis based on antigen detection

In detection of an active filarial infection or in assessment of the effectiveness of chemotherapy, the level of circulating antigen in serum will be more informative than antibody. Filarial antigen has been demonstrated in the sera and urine of infected humans and animals using antisera raised against heterologous filarial antigens from *I. carinii*, *D. immitis*, *B. malayi*, *S. digitata* (Dasgupta and Shukla Bala, 1978; Desowitz and Una, 1976; Tanabe, 1959; Dissanayake *et al.*, 1982; Hamilton *et al.*, 1984). Few attempts have been made to produce antisera against *W. bancrofti* microfilarial antigens. Administration by subcutaneous route was found to be more effective than intravenous injection in eliciting maximum immune response. Rabbit antisera against *W. bancrofti* microfilarial soluble antigens showed 2 specific precipitin bands with the corresponding antigens in agar gel diffusion (Kaliraj *et al.*, 1978; 1981a).

Circulating filarial antigen was concentrated from microfilaraemia plasma by salt precipitation and was identified as an antigen of microfilarial origin using anti rabbit m sera in CIE (Kaliraj *et al.*, 1979b). Use of immunoglobulin from chronic filarial serum (FSI) was explored for detection of circulating antigen in filarial sera and culture fluid by CIE and IHAT. FSI was found to be more efficient compared to rabbit anti mf serum in detecting circulating antigen in serum and culture fluid (Kaliraj *et al.*, 1981d; Khara *et al.*, 1981). Use of IgG fraction of FSI (FSI-G) in sandwich ELISA was found to be quite sensitive in detecting circulating antigen in 27 out of 33 microfilaraemia sera and an apparent positive correlation between the microfilarial density and the antigen titre was observed (Reddy *et al.*, 1984b). Filarial antigen was also detected in the neat urine samples of microfilaraemia patients by double antibody sandwich ELISA using FSI-C and anti rabbit urinary filarial antigen immunoglobulin and by IRMA using [<sup>125</sup>I]-rabbit IgG antibodies to *B. malayi* antigen (Reddy, M. V. R., Malhotra, A., Naidu, J. N., Hamilton, R. G. and Harinath, B. C., unpublished observations). Monoclonal antibody is another reagent with great potential for detection of specific antigen of interest. Monoclonal antibodies have been produced to heterologous filarial antigens and *W. bancrofti* mf ES antigens and are being explored for detection of filarial antigen in bancroftian filariasis.

### Immune complexes

A study of immune complexes will be of interest to understand the antigens involved and pathogenic mechanisms in disease processes. Immune complexes were determined by 3% PEG precipitation and complement consumption tests. Significant elevated

levels of circulating immune complexes (CICs) were observed in clinical filariasis compared to microfilaraemia and endemic normals. Immunofluorescence assay revealed the presence of mostly IgG and IgM immunoglobulins in the immune complexes (Prasad *et al.*, 1983a; Gajanana *et al.*, 1982). Specific filarial immune complexes have been determined by ELISA using anti C3 and filarial serum immunoglobulin-G. No correlation could be observed between the levels of CICs and clinical manifestations of the patient (Prasad and Harinath, 1984). The presence of filarial antigen in immune complex was detected by direct ELISA and the involvement of *W. bancrofti* mf ES antigen was demonstrated by competitive ELISA (Prasad *et al.*, 1983b). Antimicrofilarial ES antigen-antibody was also demonstrated in immune complexes in bancroftian filariasis by enzyme immuno assay (Prasad, 1983c).

### Field evaluation

Blood samples collected and dried on filter paper have been shown to be useful in seroepidemiological studies for detection of parasitic diseases. These filter paper blood samples are even more relevant and will be of advantage in nocturnally periodic bancroftian filariasis, where it is difficult to collect night blood smears in field studies. Malhotra *et al.* (1982) has successfully used filter paper blood samples in immunodiagnosis of bancroftian filariasis by indirect ELISA using *W. bancrofti* mf ES antigen. *W. bancrofti* mf ES antigen antibody by indirect ELISA and ES antigen by inhibition ELISA were monitored in microfilaraemia patients during diethylcarbamazine therapy. *W. bancrofti* mf ES antigen specific IgM antibody levels showed a gradual decrease in reciprocal antibody titre from pretreatment mean levels of about 15000 to 4000 at the end of the treatment (Malhotra *et al.*, 1983). However, the antigen titres during DEC therapy showed an initial increase followed by a gradual decrease during DEC treatment (Malhotra and Harinath, 1984a).

Field evaluation of ELISA using *W. bancrofti* mf ES antigen was done by screening filter paper blood samples of 462 persons residing in area endemic for bancroftian filariasis. This assay system when compared with night wet blood smear examination for microfilariae, gave a relative sensitivity of 98% and specificity of 86%. Day time blood sample can also be used in this test and thus can replace tedious night blood examination in field surveys in endemic areas (Harinath *et al.*, 1984).

Indirect ELISA using *W. bancrofti* mf ES antigen has been found to be quite useful in detection of filarial infection and in better coverage of large population in endemic areas by collecting filter paper blood samples at any time of the day in field surveys. Sensitivity and specificity of the test may be increased by including additional tests for detection of antigen or immune complexes. However, this test can not distinguish an active infection from chronic filariasis. Purification of *W. bancrofti* larval and microfilarial ES antigens by affinity chromatography and production of polyclonal and monoclonal antibodies to specific antigens should hopefully give suitable reagents for use in sensitive assays such as enzyme immunoassay and immuno-radiometric assay, providing an ideal assay system for detection of active filarial infection in the not too distant future.

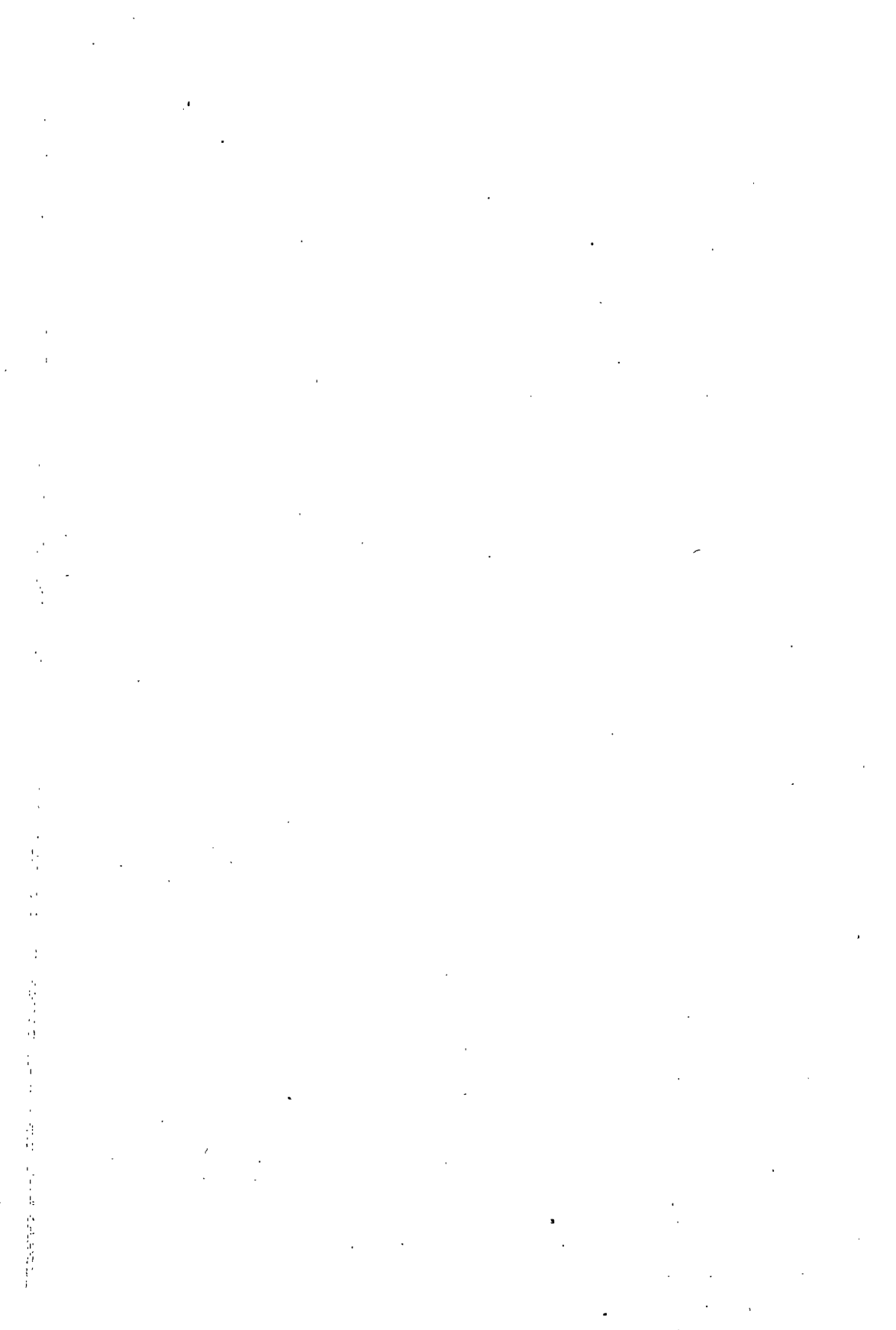
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## Role of infection in the pathogenesis of rheumatic diseases

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**Abstract.** Advanced immunological technology has revealed immunological abnormalities not only in some chronic and autoimmune connective tissue disorders but also in conditions like infective arthritis where infection apparently seems to play the only role. On the other hand role of infection in the pathogenesis of some connective tissue disorders has recently gained much importance from the observation of clinical, pathological and immunological similarities between these diseases and certain infectious diseases occurring in animal models. Meanwhile, knowledge gained into human leucocyte-A system and its association with certain diseases opens another angle in etiopathogenesis of certain rheumatic diseases. It has been postulated that adaptive mechanism of a microbe or the binding between the human leucocyte-A molecule and carbohydrate moiety of a microbe may set up an autoimmune reaction and in the presence of some triggering factors in the environment may lead on to disease manifestations. An attempt has been made to discuss the role of infection in the outcome of rheumatic diseases such as septic arthritis, polyarteritis nodosa, rheumatic fever, enteropathic arthritis, ankylosing spondylitis, rheumatoid arthritis and systemic lupus erythematoses in genetically susceptible individuals producing immunological abnormalities.

**Keywords.** Role of infection and arthritis; rheumatic diseases.

### Introduction

While the role of microbial infection in causing acute septic arthritis has already been well established, infective etiology for most of the chronic rheumatic syndromes is recently gaining much importance, based on the fact that there are striking clinical, pathological and immunological similarities between these syndromes such as rheumatoid arthritis, systemic lupus erythematoses, diffuse vasculitis etc., and certain infectious diseases occurring in animal models.

Knowledge gained into the human leucocyte antigen (HLA) system and its relation to certain diseases opens a new angle in the etiopathogenesis of some chronic and autoimmune connective tissue disorders. The immunological profile in some of the diseases such as rheumatic fever, rheumatoid arthritis and systemic lupus erythematoses suggests the possibility of immunological abnormality in these patients. Thus it is possible that in the absence of definite evidence of a specific etiological factor, infection, along with genetic susceptibility may play an important role in the outcome of many

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Abbreviations used: HLA, Human leucocyte antigen; IRR, immuno regulatory ratio; PAN, polyarteritis nodosa; AS, ankylosing spondylitis; SLE, systemic lupus erythematoses.



rheumatological disorders, producing immunological abnormalities.

Infection could be a prime factor as in the case of septic arthritis or it could be triggering factor as in the case of reactive and post infective arthritis. Research is being carried out in many centres to understand the role of infection in some of the inflammatory arthropathies such as ankylosing spondylitis, rheumatoid arthritis and systemic lupus erythematoses. This article attempts to summarise the role of infection in the etiopathogenesis of some rheumatological disorders.

## **Role of infection in rheumatic diseases**

### *General mechanism*

In acute infections the infecting organism may invade the joints or they may produce toxins or enzymes and set up an inflammatory reaction. Phagocytosis, Opsonisation due to the coating of antigen with antibodies, complement activation and the various types of hypersensitivity reactions result in the release of acute and chronic inflammatory mediators such as histamine, serotonin, Kinins, prostaglandins and lysosomal enzymes which are responsible for triggering the inflammatory process leading to tissue injury.

When the immune mechanism fails to eliminate the infecting organisms from the body, the infection persists in some sites of predilection (eg., in the reticuloendothelial cells in Brucellosis) and leads on to a chronic infective stage. Low doses of viral and other antigens have been found to induce a state of unresponsiveness of suppressor lymphocyte function in experimental animals (Fudenberg and Wellis, 1976). Under normal conditions the suppressor *T* lymphocytes check the number of normally existing auto antibody producing *B* lymphocytes, just sufficient to remove damaged or aged tissues. If, these *T* cells function is defective, the system fails to control auto antibody producing clones. A similar mechanism may play a role in the development of auto immune disorders in human beings in the presence of chronic infections with microorganisms which establish in the body due to the deficient immune mechanism of the host, defective phagocytosis and inherent capacity of some microorganisms to multiply inside the phagocytosed cells. Such a mechanism in rheumatoid arthritis is evident from the study of subtyping of *T* cells using subset specific monoclonal antibodies which show that there is an imbalance in helper/suppressor *T* cell ratio. Veys introduced the concept of immuno regulatory ratio (IRR), helper/suppressor ratio which is normally about 1.5 in peripheral blood and which may be considerably higher (above 6.0) in active rheumatoid arthritis patients (Mackenzie and Williamson, 1983). Histological appearance of synovial membrane and immunological profile of rheumatoid arthritis patients imply the continued presence of an antigen and the identification of such an antigen may well be the key to the etiology of rheumatoid arthritis.

### **Role of infection: Types of arthritis**

The organisms responsible for causing rheumatic diseases may play: (i) A direct role by invading the joints as in septic arthritis due to *Staphylococcus aureus*, *Streptococci*,

gram negative organisms etc., or (ii) An indirect role as in the case of post infective type of arthritis *eg.*, rubella arthritis and polyarteritis nodosa and reactive type of arthritis *eg.*, rheumatic fever, enteropathic arthritis due to salmonellosis, shigellosis, and yersiniosis, meningococcal infection, chickengunya infection and lyme arthritis due to tick borne spirochaetosis or the organism may play (iii) a hypothetical role in producing inflammatory poly arthropathies such as rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematoses etc.

### Role of infection in septic arthritis—Pathogenesis

Although the involvement mostly of a single joint in septic arthritis is suggestive of a local rather than a systemic pathology, the likelihood of infectious or septic arthritis is greater, if host resistance is impaired by disease or by immune suppressive drugs or where there is previous damage of an apparently normal joint by trauma or by another arthritic illness.

Except for some rare instances in which the organisms are introduced into the joint by instrumentation or penetrating wounds, invasion of joint occurs by an extension of infection from adjacent bone or soft tissue or by haematogenous spread from a focus of infection elsewhere in the body. The organisms in order to set up an infection of joints must be able to elude an active reticulo-endothelial system, to colonise synovial tissue or juxta articular bone and to penetrate into the joint cavity. Once the penetration into the joint cavity occurs, a rapid series of events occur. The synovial vessels become engorged and dilated, the synovial tissue becomes oedematous and intra-articular pressure increases due to increased volume of synovial fluid. The phagocytes which migrate into the joint try to engulf and destroy the organisms resulting in accumulation of pus inside the joints.

As mostly the infections are due to haematogenous spread, antibodies would have already been formed against the organisms and antigen-antibody complexes can be found inside the joint cavity leading on to activation of complement mediated events such as histamine release, chemotaxis and phagocytosis. Bacterial products alone even in the absence of antibody can trigger complement activation through the alternate path way (Schmid, 1979).

The impairment of host defence has been proved in instances of septic arthritis (*eg.*, diminished intracellular killing of *S. aureas* within macrophages in virus infected tissues and in subjects under cortisone therapy). Impairment of the function of polymorphonuclear cells, *viz.*, inadequate production of superoxide and hydrogenperoxide required for killing catalase negative bacteria, defects in chemotaxis and phagocytosis seen in chronic granulomatous disease have been associated with episodes of staphylococcal osteomyelitis and suppurative arthritis (Schmid, 1979).

### Role of infection—Polyarteritis nodosa (PAN)

PAN was described by Kussmaul and Maier over 100 years ago and was regarded for most of this period as a disease of unknown etiology. Presence of immune complexes of hepatitis B antigen has been demonstrated in affected tissues including kidney. Thirty

to forty per cent of patients formerly regarded as having unexplained generalised necrotising vasculitis are haepatitis *B* antigen carriers, a figure far greater than would be expected in general population. Immuno fluorescent observations led to the concept that polyarteritis may be due to the circulating antigen-antibody complexes causing lesions of vessel walls (Ziff, 1976).

### **Role of infection—Rheumatic fever**

The arthritis of rheumatic fever has been included under reactive arthritis where the infecting microbial species is well known but where the infecting organisms or the products have not yet been found at the site of lesion. Till now, the particular type of  $\beta$ -haemolytic *Streptococcus* which can unquestionably cause rheumatic fever is not yet identified even though more than 63 Griffith type of organisms have been isolated. Moreover, it is not still clear whether the rheumatic fever subjects are more susceptible to group *A* streptococcal infections or whether susceptibility lies in the unusual features of immune response.

While no significant increase in any particular human leucocyte antigen has been found by many workers a study conducted by Caughey *et al.* (1975) showed an increase in the incidence of BW17 in subjects suffering from rheumatic fever.

According to Zabriskie (1976) subjects suffering from rheumatic fever show a heightened cellular and humoral response to group *A* antigens and cross reactive autoimmunity to various cardiovascular tissue and other antigens. Kingston and Glynn (1971) have reported cross reaction of rabbit antistreptococcal sera with a number of tissue antigens including fibroblasts of heart valves, skin and synovial membrane as well as astrocytes and endothelial cells. It can be postulated that the severity and the type of target organ damage may be related to the nature of cross reacting antibodies in susceptible individuals.

### **Role of infection—Enteropathic arthritis**

Though the antecedent gut infection and the subsequent arthritis are very well known clinical entities, neither the organism nor the antigen could be demonstrated in the affected joints, but a raise in titre of antibodies against *Salmonella*, *Shigella* or *Brucella* and other such organisms could be demonstrated in serum and also in the joint fluid to a lesser extent. From the fact that the exacerbation of gut infection is closely associated with exacerbation of joint symptoms it has been postulated that the release of large quantities of antigen into the circulation from the gut predispose to the formation of soluble antigen-antibody complexes which enter the joint producing reactive synovitis by activating the classical and alternate complement pathways (Haslock, 1978).

### **Role of infection—Ankylosing spondylitis**

Ankylosing spondylitis (AS) is one among the major inflammatory rheumatic diseases. Reports from Moll (1978) and Ford (1953) suggested association between AS and

genito urinary infection. Association between AS and certain bowel diseases are also well known (McBride *et al.*, 1963). While it is possible for the spread of pelvic infection *via* the lymphatics, it is unlikely, since mostly AS occurs even before the manifestation of genito-urinary or bowel disease. The other possibility is that the infection was already established in latent form.

Brewerton *et al.* (1975) and Schlosstein *et al.* (1973) have demonstrated the evidence of genetic factors in AS (HLA B27). A cross-tolerance hypothesis has been proposed to explain the association of HLA B27 with the AS. It is proposed that the HLA molecule itself stereo chemically resembles antigens found on some external agents such as micro-organism. Damian (1964) coined the term molecular mimicry for this adaptive mechanism shown by parasites which adapt antigens of the host in an attempt to avoid detection or immunological destruction. When such microorganisms reach a state of partial adaptation whereby antibodies produced against these organisms also have antiself or autoimmune action due to resemblance to self antigen, tissue damage results by complement activation (Ebringer and Ebringer 1981). Haemagglutination and lymphocytotoxicity studies lead to the implication of *Klebsiella pneumoniae* in the pathogenesis of AS due to cross reaction with HLA B27 (Orban *et al.*, 1983).

But Welsh and Black, (1983) contradict this molecular mimicry or antigenic cross reactivity between B27 and a particular micro organism based on the observation that a variety of microorganisms precipitate a class of rheumatic disorders *viz.*, AS, Reiter's disease and reactive arthropathies which are also closely associated with HLA B27. A more likely explanation could be that for reasons of charge, or because of a lectin like property, the B27 molecule binds to carbohydrates of the microorganism thus magnifying the response against the organism.

### Role of infection—Rheumatoid arthritis

Though rheumatoid arthritis is one of the vastly studied diseases, conclusive evidence as to the etiology of this disease is still not forthcoming. Many etiological hypotheses for rheumatoid arthritis, namely, infective, metabolic, immunological etc., have been described and tested, but mostly with negative results. Genetic markers including histocompatibility antigens have not yet provided any clear evidence of an inherited genetic factor though Welsh and Black (1983) have shown a well documented association between DW4, DR4 and rheumatoid arthritis; DR4 being even more closely associated with rheumatoid arthritis than DW4.

The role of infection in causing rheumatoid arthritis is still hypothetical, although a number of workers have claimed to have isolated diphtheroids from infected synovial membrane and fluid (Duthie *et al.*, 1967). Some workers have demonstrated the presence of structures resembling the cells of *L* phase organisms in fluid cultures from rheumatoid arthritis patients (Bartholomew and Nelson, 1972).

There are persistent hints of the involvement of Rubella virus in juvenile rheumatoid arthritis (Martenis *et al.*, 1968). Elevation of para influenzae and Epstein Bar virus antibody level in juvenile polyarthritis have also been reported (Phillips *et al.*, 1973) but conclusive evidence for implicating a specific virus is lacking.

### **Rheumatoid arthritis and animal model**

Gastro intestinal abnormalities and rheumatoid arthritis like arthritis have been demonstrated experimentally in pigs which were fed on protein rich diet (Mansson and Nerberg, 1971). These animals developed joint deformities after some months and pathological changes similar to rheumatoid arthritis were demonstrable in the joints. No bacteria or mycoplasma were cultured from the joints but a delayed hypersensitivity to *Clostridium perfringens* was demonstrated along with a significant increase in the number of atypical *Clostridium perfringens* type A in intestine.

### **Rheumatoid arthritis and environment**

Recently a disease very similar to rheumatoid arthritis has been described as naturally occurring in the dogs (Alexander *et al.*, 1976). This being a domestic animal which shares a very similar environment to man could be important epidemiologically as suggested by Gottlieb *et al.* (1974) from the observation that ownership of pets can be associated with the development of rheumatoid arthritis in man. Solomon *et al.* (1975) after conducting an epidemiological survey on 964 African residence in an old established urban suburb came to the conclusion that rheumatoid arthritis is rare in rural Africans but the incidence rises with the adaptation of a more sophisticated life style.

### **Role of infection—Systemic lupus erythematoses**

Systemic lupus erythematoses (SLE) is one of the best studied examples of immune complex diseases. Despite considerable recent advances in the understanding of pathogenesis of SLE the etiology of the disease is still unknown. At the present time the disease is thought to result from a combination of genetic and infective factors.

#### *Genetic factor*

Siegel and Lee (1973) have shown from their epidemiological studies an increase in the prevalence of hyper  $\gamma$ -globulinaemia, antinuclear antibodies, biological false positive test for syphilis, clinical evidence of SLE and other connective tissue disorders in family members of patients with SLE. No definite relationship between any particular HLA group and SLE has yet been confirmed.

#### *Infective factor*

Results from animal studies have catalysed interest in possible viral etiology for SLE. C type virus particles have been associated with Canine SLE and Newzealand Mouse disease. The clinical and serological similarities between these and human SLE has stimulated a search for a viral etiology of SLE. Fresco (1970), using an electron microscope observed virus like structures in the kidney of a patient with SLE. Subsequently Gyorkey *et al.* (1972) have also described different types of inclusions in SLE glomerular epithelium. These are tubo-reticular structures found in the en-

dothelium of glomeruli. They resemble superficially the nucleocapsid of paramyxoviruses (Gyorkey *et al.*, 1972). But it is now felt that they represent cytoplasmic material, possibly altered as a result of viral injury (Graham and Houghes, 1978).

A few reported attempts at direct isolation of viruses from cell cultures of SLE tissue have yielded negative results (Feorino *et al.*, 1970). This may be due to the persistence of some viruses in joints in defective forms so that a systematic searcher for viruses in synovial membrane might have overlooked a critical population of such cells which are able to sustain a persistent infection (Denman, 1983).

Attempts to demonstrate a significant raise in any of the antiviral antibodies showed that many viral antibodies were high in SLE. Striking elevations in RNA viruses of the paramyxovirus, reo, corona and toga groups, and to DNA virus of the herpes simplex, group were often detected. This increased virus antibody in SLE can be interpreted in two ways. (i) Primary: SLE may be caused by more than one virus; (ii) Secondary: Depressed cellular immunity might allow abnormal persistence of multiple viruses or most frequent re-infection thus stimulating higher antibody levels (Phillips, 1976).

Epidemiological investigation of SLE and of drug activated SLE by Lee and Siegel (1976) suggest the concept that the SLE syndrome results from a complex interplay of factors, *viz.*, genetic, represented by familial, ethnic and sex predisposition, environmental represented by agents known to trigger activity of the disease *i.e.* ultra-violet exposure, trauma, and infection; and chemical represented by drugs capable of reacting within the body with DNA to form complexes which are more antigenic than native DNA and which more or less regularly elicit the formation of anti DNA complex antibody. Thus, the actual pathogenesis of SLE would depend on the development of anti DNA antibodies either because of alteration of DNA by means of virus, ultra-violet radiation, and drugs or marked enhancement of reactivity of immune system induced by viruses or genetic influence or a combination of any of these factors.

## Conclusion

It is obvious that environmental and genetic factors may also be important in governing the immune response and cellular susceptibility to an infection and in the clinical manifestation of the disease. Environmental agents such as drugs or sunlight may impair a balanced host microbial relationship in favour of a disease. According to Fundenburg and Wells (1976), the genetic predisposition to the autoimmune diseases merely reflects genetically determined selective immunologic deficiency for one or another micro organism.

Thus, a microbe sitting at a focal point may not be the prime cause but may be a part of a multifactorial etiopathogenic process.

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## ***In vitro* drug screening system using membrane alteration in macrophages by *Mycobacterium leprae***

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**Abstract.** The observation that live *Mycobacterium leprae* on entry into macrophages from lepromatous leprosy patients reduced the number of EA rosetting macrophages, was extended to macrophages from Swiss white mice also. Further, the fact that dead *Mycobacterium leprae* do not bring about such a change in macrophages from mice, allowed us to develop this into a bacterial viability testing system. Thus drug treated macrophages in the presence of *Mycobacterium leprae* showed normal rosetting ability if *Mycobacterium leprae* are inactivated by the drug, but showed reduced level of rosetting when bacteria were not susceptible to the drug. It was shown that a drug like dapsone, does act on *Mycobacterium leprae* based on its permeability, quantity available inside the macrophages and inhibition of its action by Para amino benzoic acid. The inactivation of *Mycobacterium leprae* by sulphone and rifampicin was also proved by the fluorescence diacetate method, which showed poorly viable bacteria after exposure to drugs. Thus it has been possible to develop a rapid drug screening method for testing the activity of unknown compound against *Mycobacterium leprae*.

**Keywords.** Macrophage; membrane changes;  $F_c$  receptors, *Mycobacterium leprae*; viability; drug screening.

### **Introduction**

With the advent of drug resistance to sulphone (dapsone di-(4-aminophenyl)-sulphone, DDS) and recently to rifampicin, two accepted drugs for leprosy, there is a need for identifying new compounds with activity against *Mycobacterium leprae*. Rapid *in vitro* screening methods are basic requirement for expanded intense drug development work. Such methods are then supplemented with *in vivo* test systems. The efforts towards identifying new compounds with activity against *M. leprae* have been insignificant, partly due to lack of rapid drug screening systems. The mouse foot pad method developed by Shepard (1960) as being adopted for routine screening work (Shepard, 1976; Rees, 1964) is time consuming. There are few *in vitro* techniques that have been described recently (Ambrose *et al.*, 1978; Nath *et al.*, 1982; Prasad *et al.*, 1981; Vithala *et al.*, 1983; Nair and Mahadevan, 1984).

We describe here, the utility of a method which uses the membrane changes induced in mice macrophages by live *M. leprae*, which leads one to distinguish the dead or

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Abbreviations used: DDS, Dapsone (di-4-aminophenyl)-sulphone; MEM, minimal essential medium; EA, erythrocyte-amboceptor; PABA, *p*-amino benzoic acid; FDA, fluorescein diacetate; EB, Ethidium Bromide.



inactivated *M. leprae* from live ones. Such an observation was made and was convincingly demonstrated by Birdi *et al.* (1983). Birdi *et al.* (1984) also described how this method could be adopted as a drug screening system using human macrophages. This communication provides further data for the usefulness of this method.

## Materials and methods

*Mycobacterium leprae* was obtained from lepromatous tissue of bacillary positive, treated or untreated patients. Bacilli were prepared as per the method of Ambrose *et al.* (1974). Such isolated bacilli were acid-fast stainable and free from other contaminating bacteria. These did not grow in normal mycobacteriological media. The bacilli were counted and  $5 \times 10^6$  bacilli were added to each Leighton tube containing macrophage cultures. *M. leprae* were also obtained from infected armadillo tissue, supplied by Dr. E. Storrs, Florida, USA. Such armadillo-derived bacteria were used as a source of drug sensitive bacteria to standardise the method of drug testing.

### Macrophage cultures

Macrophages from Swiss white mice (random bred) were obtained from the peritoneal cavity following injection of 5 ml of Eagle's minimal essential medium + 20% inactivated human serum (AB blood group) into the peritoneal cavity, after killing the animal by cervical dislocation. The peritoneal fluid was collected after agitating the cavity and 0.7 ml of the fluid was added to each presterilised Leighton tube. The macrophages obtained from the peritoneal fluid adhered to the coverslip placed inside the Leighton tube. The medium was changed every 24 h and after 3 days of such culturing, esterase positive, phagocytic cells were predominantly distributed as a uniform layer in the coverslip inside the petridish. There were no contaminating neutrophils and non adherent lymphocytes were not present in any significant numbers. All these steps were done under sterile conditions.

Seventy two hours after distribution of macrophage, the drug dapsone (Burroughs Wellcome Co., India) or rifampicin (Sigma Chemical Co., USA) was added along with the medium. The drug was washed off after 72 h exposure, following which *M. leprae* were fed to the macrophage cultures. The *M. leprae* were phagocytosed by the macrophages and were allowed to interact with the drug inside the macrophage for 72 h.

In the final step the macrophages were washed with minimul essential medium (MEM) to remove any external bacilli and then were subjected to erythrocyte-amboceptor (EA) rosetting following the method described by Birdi *et al.* (1983).

### *p*-Amino benzoic acid reversal of DDS effect

To show that DDS does enter the macrophages and its action is blocked by its well known modulator *p*-amino benzoic acid (PABA) the following experiment was done.

The macrophage culture from the peritoneum of Swiss white mice was cultured and after 72 h of culturing it was exposed to drug DDS and PABA. The compounds were allowed to enter the macrophage for another 72 h following which a wash with MEM

was given and *M. leprae* was added. The *M. leprae* inside the macrophage were allowed to interact with the compounds for further 72 h. Following the incubation period the macrophages were washed with MEM to remove excess bacilli and were then subjected to EA rosetting.

#### *Estimation of DDS inside the macrophages*

The macrophages grown *in vitro* for 72 h, were treated as described above with the drug dapsone (DDS) following which they were harvested by scraping them with a rubber policeman. The macrophages were then washed three times with saline so as to get rid off extra cellular DDS. The count of the macrophages was taken in a haemocytometer after suspending the macrophages in 1 ml saline.

The macrophages were then lysed by subjecting them to 8 cycles of freeze-thawing. Redistilled ethyl acetate (6 ml) (checked for fluorescence quenching) was added to the lysate in the presence of 0.5 ml 10 N NaOH. The mixture was vortexed to facilitate the extraction of DDS from lysate to ethylacetate. After separation of the solvent and aqueous phase (20 min), the ethylacetate phase was aspirated out into another tube with a pasteur pippette. To the ethylacetate phase 0.5 g of NaCl was added to remove off any water molecules carried during the extraction. The ethyl acetate phase was then allowed to separate from the NaCl which sedimented at the bottom. The ethylacetate phase was transferred to another tube and then checked for fluorescence using a spectrofluorimeter (SFM23-Kontron) which had already been standardised to ng levels of DDS, with a sensitivity upto 2 ng/ml. The wave length settings were 285 nm for excitation and 350 nm for emission. There was always a control culture of macrophages which had not been exposed to the drug to compare with the macrophage culture which was exposed to the drug. The results were computed as follows to eliminate the background fluorescence of macrophage itself (Peters *et al.*, 1981).

DDS (in ng)/million macrophages is concentration that causes an absorption level of 1 million macrophages exposed to drug minus absorption level of 1 million macrophages not exposed to drug. The quantitation of the drug was determined from a standard curve of fluorescence absorption determined for various DDS standard concentrations.

#### *Determination of viability of M. leprae within macrophages*

The method used was the fluorescein diacetate (FDA)/ethidium bromide (EB) (Both from Sigma Chemical Co., USA) staining of *M. leprae* (Kvach *et al.*, 1982 as modified in our laboratory by Nalini, R., Alaka, B. and Mahadevan, P. R., Personal communications).

One set of coverslips with adherent macrophages was processed in the manner described above with drug and bacilli following which they were checked for viability.

The coverslips on the day of termination were rinsed in MEM to wash off the excess of bacilli and placed over a drop of the staining solution (20 µg of FDA and 20 µg EB in 5 ml of phosphate buffered saline). This was incubated for 29 min in the dark. The coverslip was sealed with nail polish to prevent evaporation and observed under incident fluorescent ultra-violet light using Carl Zeiss fluorescence microscope at a magnification of 400.

A total number of 200 macrophages were counted and the number of macrophages with green bacilli (denoting live *M. leprae*) were calculated. The total number of macrophages with bacteria were estimated by determining their level with acid fast bacteria. The viability is then indicated in the number of macrophages showing green fluorescing bacteria to the total number of acid fast bacteria containing the macrophages.

## Results

The entry of the drug DDS into the macrophages was clearly demonstrable by an estimation of the level of DDS in purified macrophages. The internal concentration of DDS increased with increasing level of DDS outside the cells (table 1) and it has been possible to calculate the DDS concentration/ $10^6$  macrophages.

**Table 1.** DDS uptake by macrophages when exposed to various levels of the drug.

DDS added in the medium		DDS incorporated ng/ $10^6$ macrophages (three separate experiments)		
ng/tube	ng/ml	1	2	3
10	14.2	1.6	1.7	1.6
20	28.5	2.1	2.5	2.6
50	71.4	5.2	7.1	5.1
100	142.8	8.1	8.5	8.2

The ability of macrophages from the peritoneal cavity of mice to reveal the  $F_c$  receptors as monitored by EA rosetting is clearly reduced in the presence of phagocytosed live *M. leprae* (table 2). If however the bacteria were killed or inactivated before phagocytosis then the rosetting ability is the same as the control. Since *M. leprae* are susceptible to DDS, the drug treated macrophages show rosetting ability as good as the control, indicating that the drug has inactivated the phagocytosed *M. leprae* (table 2). The data presented in table 3 show a similar observations using rifampicin at a concentration of 5  $\mu$ g/Leighton tube.

To demonstrate that the action of the drug on *M. leprae* is a true feature, an experiment was done with various concentrations of DDS so as to decide the level at which *M. leprae* was inactivated. Data presented on table 4 showed that at concentrations below 20 ng/ml in the medium, the bacteria inside the macrophages were not inactivated, and thus the control level of EA rosetting ability of the macrophages is not restored. Thus a clear concentration dependence was demonstrable.

PABA a known inhibitor of sulphone could reverse the effect of DDS on *M. leprae* in those macrophages that were given both DDS and PABA (table 5). A degree of antagonism has been thus demonstrated.

The drug exposed *M. leprae* that were inactivated and was responsible to show the normal level of EA rosetting of the macrophages, could also be shown to be nonviable

**Table 2.** Per cent EA rosetting macrophages in presence of armadillo derived *M. leprae* with and without exposure to dapsons.

No. of the Expt.	Control (Mθs only)	DDS only	Macrophages with		Heat killed <i>M. leprae</i> only
			Live <i>M. leprae</i> only	DDS and <i>M. leprae</i>	
1	53	40	25	51	59
2	38	36	23	38	49
3	50	40	34	51	58
4	62	44	25	53	64
5	66	46	33	52	66
6	60	40	34	61	68
7	64	42	37	62	69
8	59	48	34	60	68
9	57	46	26	58	66
10	56	39	42	54	55
11	54	41	41	56	52
Mean ± S.D.	56 ± 2	42 ± 1	32 ± 2	54 ± 2	61 ± 2

Control: *M. leprae* added culture significant  $P < 0.05$  *M. leprae* only: Drug + *M. leprae* significant  $P < 0.05$ .

**Table 3.** Per cent EA rosetting macrophages in presence of armadillo derived *M. leprae* with and without exposure to rifampicin.

No. of the Expt.	Control (only Mθs)	Rifampicin only	Macrophages with		Heat killed <i>M. leprae</i> only
			Live <i>M. leprae</i> only	Live <i>M. leprae</i> and rifampicin	
	A	B	C	D	E
1	49	32	29	70	66
2	50	35	35	73	60
3	54	32	34	70	65
4	56	34	37	71	69
5	70	54	34	69	68
6	65	49	26	68	67
Mean	57 ± 4	39 ± 4	33 ± 2	70 ± 1	66 ± 2

P value A - C < 0.05 Significant

P value D - C < 0.05 Significant

by the fluorescent staining method. The data presented in table 6 clearly show that on exposure of *M. leprae* to a level 100 ng (0.1 µg/ml) of DDS caused the *M. leprae* to become nonviable; since such drug treated macrophages showed reduced number of macrophages with green fluorescent bacteria; but showed acid fast stainable bacteria in much great numbers. The drug untreated macrophages containing viable *M. leprae* were seen as green bacteria fluorescing in higher number of macrophages and comparable well with macrophages containing acid fast bacteria. Similar results were

**Table 4.** Per cent of macrophage exhibiting EA rosetting in presence of *M. leprae* and exposed to various concentration of DDS.

DDS added per tube	Concentration of DDS (ng/ml medium)	Control macrophages only	Live <i>M. leprae</i> only	Macrophages containing		
				DDS only	DDS and live <i>M. leprae</i>	Heat killed <i>M. leprae</i>
		A	B	C	D	E
10	14.2	49 ± 14	24 ± 6	30 ± 8	31 ± 9	55 ± 13
15	21.4	70 ± 7	46 ± 7	59 ± 3	59 ± 3	70 ± 0
20	28.5	75 ± 5	48 ± 6	65 ± 5	76 ± 5	75 ± 5
50	71.4	72 ± 2	46 ± 8	60 ± 2	71 ± 2	72 ± 2
100	142.8	68 ± 3	42 ± 3	56 ± 9	70 ± 3	69 ± 5

Each value is an average of 4 experiments with each concentration of the drug added.

A - B  $P < 0.05$  significant.

A - C  $P < 0.05$  -do-

A - D  $P < 0.05$  significant upto 15 ng only.

A - E  $P > 0.05$  not significant.

A - D  $P < 0.05$  significant above 15 ng only.

**Table 5.** Effect of PABA in relation to DDS action on *M. leprae*.

Per cent EA rosetting macrophages					
Control	Live <i>M. leprae</i>	PABA	Macrophage with		
			PABA + live <i>M. leprae</i>	DDS + live <i>M. leprae</i>	DDS + PABA + live <i>M. leprae</i>
60	33	45	51	59	28
85	48	66	50	84	68
88	58	74	62	84	74

Dosage DDS - 1 µg/tube - 1.42 µg/ml.

PABA - 0.5 mg/tube - 0.70 mg/ml.

obtained by using rifampicin as the drug (table 6). In such an experiment the EA rosetting ability was also well correlated. The viable bacteria (green fluorescent) containing macrophages in drug treated samples can be contrasted with number of macrophages containing acid fast bacteria to show the loss of viability in the presence of the drug since acid fast staining give total bacterial load and green fluorescent bacteria indicate only viable among them.

## Discussion

The earlier observation by Birdi *et al.* (1983), that live *M. leprae*, on entry into the macrophages cultured *in vitro* from human peripheral blood, could reduce the number of EA rosetting macrophages, has been found to be true with the peritoneal macrophages from Swiss white mice. Like, in the human system, heat killed *M. leprae*

**Table 6.** Correlation of viability of *M. leprae* as determined by fluorescent diacetate method (FDA/EB) with EA rosetting ability of macrophages and *M. leprae* in presence and absence of drugs.

Percent macrophages having phagocytosed <i>M. leprae</i> (acid fast staining)	Per cent EA rosetting macrophages		Viability by FDA/EB			
	Control	With live <i>M. leprae</i>	With DDS/rifampicin and <i>M. leprae</i>		With live <i>M. leprae</i>	With DDS and <i>M. leprae</i>
	DDS		0.1 µg/tube	0.5 µg/tube	0.1 µg/tube	0.5 µg/tube
78	80	41	78	81	61	36
50	74	38	73	75	47	29
	Rifampicin					
85	79	49	80*	88**	39	28*
75	56	40	59	70	44	21

\* 0.2 µg/tube

\*\* 0.5 µg/tube rifampicin

could not do so. Birdi *et al.* (1984) had projected the usefulness of this system to screen drugs active against *M. leprae* by demonstrating the activity of rifampicin on *M. leprae* using EA rosetting method with human macrophages. The data presented in this paper show the adaptation of the method by a standardised procedure using macrophages from mice and armadillo derived *M. leprae*. Entry of DDS into macrophages has been clearly demonstrated and estimation of DDS inside the macrophages could also be done. The concentration effect of DDS to inactivate the *M. leprae* was also indicated by the fact that DDS treated *M. leprae* did lose their viability which was further proven by the loss of their ability to pick up fluorescent labelling after treatment with fluorescent diacetate (Kvach, 1982). That the DDS is acting through the recognised pathway of a sulpha drug against *M. leprae* (Seydel, 1980) was further demonstrated by the partial reversal of DDS activity by PABA. Besides DDS, rifampicin a known anti *M. leprae* agent could also be shown to be active against *M. leprae*, ingested inside the macrophages, by the loss of their ability to pick up fluorescent labelling after treatment with fluorescent diacetate (Kvach, 1982). This was also shown by monitoring the ability of the bacteria to reduce the number of EA rosetting macrophages in the presence of the drug.

Several clinically isolated *M. leprae* have been tested for drug sensitivity and resistance to DDS and rifampicin using this system. They are also being correlated with behaviour of the isolates in mice foot pad (data not given).

Thus it is clear that by using macrophages and armadillo derived *M. leprae*, the drug sensitivity of the bacteria to known and unknown drugs could be determined using the EA rosetting ability of the macrophages. The test system can be completed in a period of 8–9 days, the shortest time needed in any of the test systems described so far (Vithala *et al.*, 1983; Prasad *et al.*, 1981; Nath *et al.*, 1982) and quite similar to the one described from our earlier work using the cholesterol metabolism of the macrophages (Nair and Mahadevan, 1984).

Several derivatives of methoxyindole have been tested using the method described

here and some of them were found to be effective against *M. leprae* (data not given).

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## Monoclonal antibodies against *Wuchereria bancrofti* microfilarial excretory-secretory antigens

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**Abstract.** A battery of monoclonal antibodies were produced against *Wuchereria bancrofti* microfilarial excretory-secretory antigens and their specificity was studied using different filarial antigens. Among the 1116 wells plated out, 42 % of the wells developed hybrids and 5 % of the hybrids showed anti *Wuchereria bancrofti* microfilarial excretory-secretory antigens. Specificity studies on the antibodies produced from 63 cloned and expanded hybrids showed 10 clones which were specifically positive only to *Wuchereria bancrofti* microfilarial excretory-secretory antigens.

**Keywords.** *Wuchereria bancrofti*; excretory-secretory antigens; monoclonal antibodies; enzyme linked immunosorbent assay.

### Introduction

The application of hybridoma technology has a great potential in immunoparasitology to produce monoclonal antibodies of interest in diagnosis and immuno prophylaxis. Yoshida *et al.* (1981) and Potocnjak *et al.* (1980) have demonstrated the utility of monoclonal antibodies in the protection of mice against malarial infection. In schistosomiasis Grzych *et al.* (1980) have produced monoclonal antibodies demonstrating *in vitro* cytotoxicity against schistosomules. Mitchell *et al.* (1983) and Cruise *et al.* (1981) have used selected monoclonal antibodies in the development of immunodiagnostic assays for schistosomiasis infection. des Moutis *et al.* (1983) have used monoclonal antibodies in detection of circulating antigen in onchocerciasis patients. Studies from our laboratory (Kharat *et al.*, 1982; Malhotra *et al.*, 1982 and Harinath *et al.*, 1984) have shown that *Wuchereria bancrofti* microfilarial excretory-secretory antigens (*Wb* mf ES Ag) were superior to somatic antigens in terms of specificity and sensitivity when used in immunodiagnosis of bancroftian filariasis. This communication presents successful production of monoclonal antibodies to *Wb* mf ES Ag and studies on their specificity against different filarial antigens.

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Abbreviations used: *Wb* mf ES Ag, *Wuchereria bancrofti* microfilarial excretory-secretory antigens; DMEM, Dulbecco's Modified Eagles Medium; i.p., intraperitoneally; ELISA, enzyme linked immunosorbent assay; BSA, bovine serum albumin; DEA, diethionolamine; *Di* Ag, *Dirofilaria immitis* Ag; *Bm* mf Ag, *Brugia malayi* mf Ag; *Al* Ag, *Ascaris lumbricoides* Ag; *Sd* 2-4 Ag, *Setaria digitata* 2-4 Ag.



## Materials and methods

### *Cells and media*

The plasmacytoma cell line used in the fusion was Balb/c mouse derived, non secreting and azaguanine resistant (NS-1). Dulbecco's Modified Eagles Medium (DMEM, of GIBCO Laboratories, USA) supplemented with 10% fetal calf serum (K. C. Biologicals), azaguanine (0.1 mM), L-glutamine (4 mM) and gentamycin (0.1%) was used to maintain the parental cell line (NS-1). The hybrids were grown in OPI-HAT medium *i.e.* DMEM supplemented with 10% NCTC 109 medium (M. A. Bioproducts), 15% fetal calf serum, bovine insulin, pyruvate, oxalo-acetate, hypoxanthine, thymidine and aminopterin. The same medium without aminopterin (OPI-HT medium) was used to maintain the cloned hybrids.

### *W. bancrofti microfilarial excretory-secretory antigens*

*Wb* mf ES Ag was prepared as described by Kharat *et al.* (1982). The microfilariae were isolated by nucleopore membrane filtration (5  $\mu$ m pore size) from night blood samples of microfilaraemia cases and were maintained *in vitro* in medium 199 supplemented with organic acids and sugars of Grace's medium but without serum. The culture fluid was collected after every 24 h, centrifuged at 13000 *g* at 4°C for 30 min and concentrated 200 fold by ultrafiltration and freeze-drying. The protein was estimated using Biorad protein assay kit.

**Immunization schedule:** Eight Balb/c mice were injected intraperitoneally (i.p.) with 10  $\mu$ g of *Wb* mf ES Ag in complete Freund's adjuvant (Grand Island Biological Company, USA). Second and third booster doses were given (i.p.) with 10  $\mu$ g of antigen in incomplete Freund's adjuvant in the 4th and 5th weeks. Filarial antibody in the serum of the immunized animals was monitored by enzyme linked immunosorbent assay (ELISA) and all the animals were showing the presence of filarial antibody (1:40 and above reciprocal of antibody titre). Last booster dose was given (i.p.) with 10  $\mu$ g of antigenic preparation without any adjuvant in the eighth week and four days later spleens were removed for fusion.

**Cell fusion and selection of hybrids:** The procedure followed was as described by Kennett *et al.* (1978). The immune mice were sacrificed by cervical dislocation and the spleens were aseptically removed. Cells were removed from spleens by teasing them with two sterile forceps. Erythrocytes were removed by lysis with 0.85% ammonium chloride solution for 2 min. The cells from each spleen were mixed separately with an equal number of NS-1 myeloma cells and they were pelleted together in a 50 ml round bottomed tube (Falcon). After removing the supernatant, the pellet was resuspended and 0.5 ml of fusion medium (50  $\mu$ l DMSO, 350  $\mu$ l polyethylene glycol 1000 and 650  $\mu$ l DMEM) was added and the cells were centrifuged at 400 *g* at room temperature for 6 min. Hybrids and free cells were resuspended in 3 ml DMEM without serum and the final volume of the cell suspension was made with OPI-HT medium (26 ml to a spleen of  $10^8$  cells). The cells were plated out into 96 well flat bottomed tissue culture plates (Costar, Cambridge, Mass.). The plates were incubated at 37°C in a humidified atmosphere of 8% CO<sub>2</sub> in air. The next day, 1 drop of HT medium with 2X

aminopterin was added to each well to initiate the selection of hybrids. Four days later the HT medium was changed twice with an interval of 2 days between each change.

**Cloning and expansion of hybrids:** The hybrids which were consistently positive in ELISA for the production of anti *Wb* mf ES Ag antibodies were cloned by limiting dilution method in 96 well flat bottom micro culture plates (Costar), containing  $\gamma$ -irradiated (5000 rads) normal mouse spleen cells as feeder layers. When the clones became macroscopically visible, ELISA was run with the supernatants from 12 wells of the plate with 100 cells/well concentration. Depending upon the yields from these wells, 10 to 50 wells from 1 cell/well plate were screened in ELISA, and 2-4 positive clones from 1 cell/well plate were expanded for further analysis and storage.

**Enzyme linked immunosorbent assay for screening culture supernatants:** Indirect ELISA was done as described by Voller *et al.* (1979) for screening culture supernatants. Fifty  $\mu$ l of *Wb* mf ES Ag or other antigens diluted in 0.06 M carbonate buffer pH 9.6 were added to the wells of flat bottom PVC microtitre plates (Falcon) and incubated overnight at 4°C. After draining out the antigen solution, the wells were blocked by washing the plate three times with 0.5% bovine serum albumin (BSA, cohn Fraction V, Sigma) in carbonate buffer. The plate was further washed 4 times with 0.01 M phosphate buffered saline pH 7.2 containing 0.05% tween 20 (PBS/T). Fifty  $\mu$ l of neat culture supernatants from hybrids or NS-1 cells were added into the wells and incubated for 2 h at room temperature. After washing the plate 4 times with PBS/T, 50  $\mu$ l of alkaline phosphatase labelled to affinity purified anti mouse IgG + M (Boehringer Mannheim Biochemicals) was added to the wells and incubated for 2 h at room temperature. Followed by final washing with PBS/T, 50  $\mu$ l of the substrate consisting of *p*-nitrophenyl phosphate (Sigma chemical Co. USA) in diethanolamine (DEA) buffer pH 9.8 (5 mg tablet/5 ml DEA buffer) was added into wells. The optical density was measured by Titertek multiscanner (FLOWLABS) at 405 nm.

#### *Characterization of the specificity of the immunoglobulins produced*

The specificity of monoclonal antibodies was assessed by measuring their reactivity with *Wb* mf ES Ag, 5 other filarial antigens and one non-filarial helminthic antigen. The *Wb* mf soluble Ag was prepared as described by Kaliraj *et al.* (1978) and the *Wb* circulating filarial antigen-2 was prepared as described by Reddy and others (Reddy, M. V. R., Prasad, G. B. K. S. and Harinath, B. C., unpublished data). *Dirofilaria immitis* antigen (*Di* Ag), *Brugia malayi* mf Ag (*Bm* mf Ag), *Setaria digitata* 2-4 antigen (*Sd* 2-4 Ag) and the *Ascaris lumbricoides* adult worms Ag (*Al* Ag) were obtained from Prof. Willy F. Piessens and Izaskun petrelanda of Harvard School of Public Health, Boston, USA. All the antigens were coated on to microtitre plates (Falcon) at a concentration of 5  $\mu$ g/ml except *Sd* 2-4 Ag which was coated at a concentration of 2  $\mu$ g/ml. Negative controls included the supernatants screened in the wells coated with BSA alone and NS-1 cell culture supernatant incubated in the antigen coated wells.

#### **Results and discussion**

Among 1116 wells plated out in 2 experiments of cell-fusion, 42% of the wells showed the development of hybrids. When the supernatants from these were tested for anti-*Wb*

mf ES Ag activity in indirect ELISA, 5% of the hybrids showed antibody production. Hybrids from all the wells secreting anti *Wb* mf ES Ag antibodies were cloned, and 2-4 positive clones from each of the positive hybrids were selected for expansion and further studies.

Table 1 shows the results of the specificity studies on the antibodies produced by the expanded 63 clones using *Wb* mf ES Ag, 5 other filarial antigens and non filarial helminth antigen. The supernatants of the clones showing optical density two times or more than the reactivity in the wells coated with BSA alone were taken as positive. Based on these criteria 33 out of 63 clones remained positive (to secrete monoclonals) to *Wb* mf ES Ag. *Wb* mf soluble Ag, and *Al*. Ag had 19 clones secreting positive monoclonal antibodies to each of them. The other 4 filarial antigens, *Wb* circulating filarial Ag-2, *Bm* mf Ag, *Di* Ag, and *Sd* 2-4 Ag had 7, 1, 12 and 2 positive clones respectively. Ten clones (*Wb* E 9, *Wb* E 17, *Wb* E 24, *Wb* E 25, *Wb* E 26, *Wb* E 33, *Wb* E 34, *Wb* E 35, *Wb* E 36 and *Wb* E 37) were specifically positive only to *Wb* mf ES Ag (table 2).

Table 1. Specificity of the monoclonal antibodies secreted by 63 expanded clones in indirect ELISA using different antigens.

Antigen	No. of clones* positive
<i>Wb</i> mf ES Ag	33
<i>Wb</i> mf soluble Ag	19
<i>Wb</i> circulating filarial Ag-2	7
<i>Di</i> Ag	12
<i>Bm</i> mf Ag	1
<i>Sd</i> 2-4 Ag	2
<i>Al</i> adult Ag	19

\* Clones with optical density values more than twice to their reactivity in the wells coated with BSA only.

Studies on the diagnostic utility of *Wb* mf ES Ag (Kharat *et al.*, 1982, Malhotra *et al.*, 1982; Harinath *et al.*, 1984) have shown that the *Wb* mf ES Ag is highly useful in immunodiagnosis of filariasis. Two antigen fractions reacting differently with different stages of infection have been isolated from *Wb* mf ES Ag by ultramembrane filtration (Reddy *et al.*, 1984). It will be of interest to characterize the *Wb* mf ES Ag to understand their importance in diagnosis and in anti-parasite immunity of the host. But our previous attempts to raise precipitative antibodies to *Wb* mf ES Ag in rabbits were unsuccessful (Reddy, M. V. R. and Harinath, B. C. unpublished data). However from this study using *Wb* mf ES Ag, the first one as far as we are aware, it can be envisaged that monoclonal antibodies can be raised *in vitro* against *Wb* mf ES Ag (table 2). The monoclonal antibodies thus produced have great potential in immunodiagnostic studies and in characterizing the ES antigens. The studies are in progress in that direction.

Table 2. Relative reactivities of 10 *Wuchereria bancrofti* microfilarial excretory-secretory antigen monoclonal antibodies with other filarial and non-filarial antigens.

Clone code No.	Optical density values (405 nm) in ELISA with									
	Wb mf ES Ag	Wb mf S Ag	Wb CFA-2 Ag	Di Ag	Bm mf Ag	Sd2-4 Ag	Al ad Ag	BSA		
Wb E9	1.13	0.86	0.6	0.46	0.70	0.65	0.75	0.5		
Wb E17	0.86	0.58	0.52	0.42	0.57	0.50	0.67	0.43		
Wb E24	0.12	0.04	0.02	0.04	0.00	0.00	0.06	0.04		
Wb E25	0.14	0.04	0.01	0.05	0.00	0.00	0.01	0.04		
Wb E26	0.12	0.03	0.01	0.05	0.00	0.00	0.05	0.03		
Wb E33	0.746	0.46	0.23	0.24	0.50	0.43	0.35	0.36		
Wb E34	0.63	0.45	0.26	0.21	0.40	0.38	0.30	0.31		
Wb E35	0.11	0.04	0.04	0.07	0.00	0.02	0.08	0.05		
Wb E36	0.10	0.06	0.04	0.07	0.02	0.02	0.08	0.05		
Wb E37	0.10	0.07	0.04	0.06	0.00	0.04	0.06	0.04		

Wb mf S Ag, *Wuchereria bancrofti* microfilarial soluble antigen; Wb CFA-2 Ag, *Wuchereria bancrofti* circulating filarial antigen-2; Sd2-4-*Setaria digitata* antigen-24; Al ad Ag, *Ascaris lumbricoides* adult antigen.

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## Humoral immune response to filarial antigens in chyluria

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**Abstract.** Humoral immune parameters like total immunoglobulins and specific antibody levels in serum were studied in filarial chyluria patients. Mean serum IgG was significantly reduced in this group compared to normal controls, while IgA and IgM levels remained comparable to controls. Anti-filarial antibody titre as measured by enzyme-linked immunosorbent assay also was significantly reduced. However, the total and specific IgE antibody titre was similar to that of controls. Specific IgE contents of the patients' sera could be related to their microfilaraemic status.

**Keywords.** Chyluria; humoral immunity; IgE.

### Introduction

Chyluria is one of the many clinical manifestations of filariasis. It is the commonest of the chylous complications of lymphatic obstruction. Several studies in the past have analysed the cellular and humoral immune parameters of lymphatic filariasis patients (Desowitz *et al.*, 1976; Paranjape, 1981). Until recently, there have been very few published reports on the status of cellular and humoral immunity in patients with chyluria. We have in our previous experiments observed a reduction in general cellular immune response (Raja *et al.*, 1983). The present paper summarises our findings regarding the total and specific immunoglobulin levels and reaginic antibody response in these patients.

### Materials and methods

Patients for the study were chosen from those attending the Filariasis Clinic of Government General Hospital, Madras. All the patients had the complaint of passing milky urine. Five of the patients had circulating microfilaria in their blood, 6 of the

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Abbreviations used: SRID, Single radial immunodiffusion; ELISA, enzyme linked immunosorbent assay; PRIST, paper radio immunosorbent test; DiA, *Dirofilaria immitis*; BmA, *Brugia malayi*; SeA, *Setaria digitata*; RAST, radio allerge sorbent test.

patients had other clinical manifestations of filariasis like lymphadenitis, lymphangitis and hydrocele and 7 did not have any evidence of filariasis. The male : female ratio was approximately 5 : 1 and they were spread over a broad age group ranging from 18 yrs to 50 yrs. Lymphangiography was done for all the patients. Dilated tortuous lymphatics and retrograde flow at several sites were observable.

#### *Total protein estimation*

Total protein estimation in serum was done according to Lowry *et al.* (1951).

#### *Immunoglobulin estimation*

IgG, IgA and IgM in serum were estimated by single radial immuno diffusion (SRID) following Rowe *et al.* (1970). Serum IgE was estimated by paper radio-immunosorbent test (PRIST). (Kit from Pharmacia Diagnostics, New Jersey, USA).

#### *Specific antibody measurement*

Specific antihelminthic antibodies in serum of class IgG were measured by enzyme linked immunosorbent assay (ELISA). Helminthic antigens *Dirofilaria immitis* (DiA), *Brugia malayi* (BmA), and *Setaria digitata* (SeA) were used. DiA and BmA were saline extracts of the adult worms obtained from the Laboratory of Parasitic Diseases, NIAID, NIH, USA. SeA was an extract of the adult worms in tissue culture medium RPMI 1640.

The sera from normals and patients were tested at a dilution of 1/800. The assay was done according to the method of Voller *et al.* (1976) with slight modifications as reported elsewhere (Narayanan *et al.*, 1983).

Specific IgE antibodies were measured by radio-allergo sorbent test (RAST) using BmA as the antigen (Hamilton *et al.*, 1981).

### **Results**

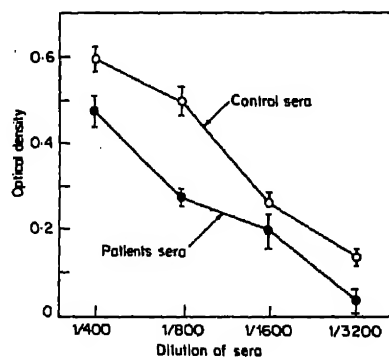
Immunoglobulins G, A and M in 17 chyluria sera have been measured using SRID. Twenty eight normal sera were also included as controls. The results are presented in table 1. Total proteins, IgA and IgM, all remained comparable in chyluria and normal control sera. The mean IgG levels in the serum of chyluria patients was  $790 \pm 446$  mg/dl, whereas that of normal subjects was  $1295 \pm 414$  mg/dl. This reduction in the IgG levels was statistically significant ( $P < 0.001$ ).

Anti-helminthic IgG antibody levels were estimated using ELISA. Figure 1 shows the mean antibody titres in the patients' sera and that of endemic controls using BmA antigen. The mean antibody levels in the patient group was lower than the endemic controls. Similar reduction was seen also with heterologous antigens of DiA and SeA (figure 2).

Serum IgE levels have been estimated using PRIST in 15 patients and 10 normal subjects. Geometric mean of total IgE level in the patient group was approximately 10 times higher than the normal control group. BmA specific IgE levels in the patients as measured by RAST were comparable to the normal control sera with mean values of

**Table 1.** Total proteins and immunoglobulins in chyluria sera.

Patients S. No.	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)
1	837	195	177
2	804	446	163
3	609	428	83
4	609	184	163
5	364	148	55
6	773	180	76
7	185	267	150
8	287	850	55
9	520	108	42
10	358	37	138
11	499	50	376
12	1803	286	142
13	1484	213	165
14	1377	203	213
15	1097	66	190
16	914	85	225
17	914	96	169
Mean $\pm$ S.D.	790 $\pm$ 446	226 $\pm$ 199	151 $\pm$ 80
Mean $\pm$ S.D. NHS (28)	1295 $\pm$ 414	172 $\pm$ 72	192 $\pm$ 95
<i>t</i>	3.85	1.29	1.46
<i>p</i>	< 0.001	> 0.20	$\approx$ 0.20

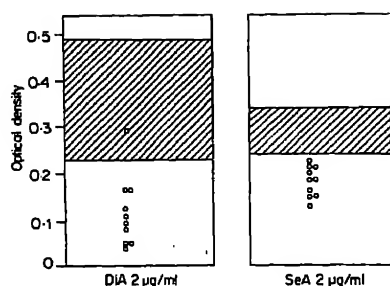
**Figure 1.** Anti BmA antibody levels of chyluria patients and control subjects.

54 ng/ml for patients and 77 ng/ml for controls. The level of specific IgE was less in microfilaraemic patients than the microfilaria negative group (table 2).

## Discussion

Total serum IgG levels in chyluria patients was significantly lower than that of normal subjects. The total serum proteins, IgM and IgA levels of chyluria sera were within the





**Figure 2.** Levels of antifilarial antibody to heterologous antigens in chyluria sera.

Antibody levels have been compared at a dilution of 1/1600 of the sera. Hatched area represents the mean  $\pm$  S.D. of the normal sera.

**Table 2.** Total and specific IgE in chyluria sera.

S. No.	Total IgE ng/ml	BmA specific IgE ng/ml
1*	2880	27
2	2400	58
3*	2016	80
4	5760	52
5	9120	43
6	3240	28
7*	2400	80
8	3024	14
9*	8640	65
10†	8160	62
11†	7680	66
12	3366	66
13	11040	55
14	18720	254
15	5640	57
GM for patients	5057	54
GM for 10 controls	636	77
GM for mf + ve group	3780	37
GM for mf -ve group	6620	73

\* Microfilaria positive patients.

† Microfilariaemic status could not be ascertained.

normal range. Many investigators have reported, either an increase in the IgG level of sera of filarial patients (Desowitz *et al.*, 1976; Saha *et al.*, 1979) or normal level of IgG (Paranjape, 1981). In our patients, the mean serum IgG was significantly reduced than in normals. But out of the 17 patients studied, only 5 showed drastic reduction in IgG, whereas the others remained within the normal limits. Date *et al.* (1981) had also reported normal IgG levels in chyluria patients. The reduction in the level of IgG observed in some patients could be due to the loss of proteins from retroperitoneal lymphatics into the urinary tract occurring as a result of lymphatic urinary tract communication. This was evidenced by the lymphangiograms. Similar bulk loss of immunoglobulins into the gastro-intestinal tract and resultant hypogammaglobu-

linaemia in serum was encountered in patients with intestinal lymphangiectasia (Strober *et al.*, 1967). In 4 of the chyluria patients' urine analysed, considerable quantity of IgG and other proteins were detected, strengthening the possibility that the leakage may be the main causative factor for hypogammaglobulinaemia of serum. However, additional factors like decreased synthesis or increased catabolism may also play a role.

Nonavailability of adult *Wuchereria bancrofti* for antigen preparation, necessitated the use of other antigens prepared from human (BmA) and non-human worms (DiA and SeA) which have been shown to cross-react extensively with *W. bancrofti* (Takahashi and Sato, 1976; Dissanayake and Ismail, 1980; Tandon *et al.*, 1981). These antigens could be obtained comparatively easily from animal hosts. Mean antifilarial antibody levels in general in these patients were significantly reduced in comparison with endemic normal controls. The controls themselves showed detectable level of antibody activity because they were also sensitized to the endemic helminthic infections. But the response shown by the chyluria patients was far lower than the controls themselves, which was in accordance with the reduced serum IgG levels. However, reaginic sensitization to the filarial parasite was not hampered in chyluria patients. Mean total IgE was 10 times more than that of the normals and antigen specific IgE remained similar to the normals. Such non-specific potentiation of IgE response has been reported earlier in other helminthic diseases (Turner *et al.*, 1979). A distinct relationship could be discerned between filarial specific IgE and parasitological status of the patients. Mean BmA specific IgE was 73 ng/ml in the microfilariae negative group and 37 ng/ml in the microfilariae positive group.

The decreased IgE level in the microfilariaemic patients could be due to the absorption of the specific IgE onto the surface of the microfilariae. Evidence for such an absorption has been shown by Mehta *et al.* (1980), where they have demonstrated the adherence of spleen cells to the microfilariae in the presence of immune serum and that the active principle in the immune serum responsible for cellular adhesion was IgE. The reduction may also be due to a specific suppression of production of IgE in the presence of microfilariae. Similar low level of specific IgE had been observed in other non-chyluria microfilariaemics also (Hussain *et al.*, 1981).

### Acknowledgements

The authors are thankful to Prof. S. Subrahmaniam, Head of the Department of Microbiology, Post-Graduate Institute of Basic Medical Sciences, Madras; Dr. V. Kumaraswamy, Tuberculosis Research Centre, Madras and Prof. K. V. Thiruvengadam, Head of the Department of Medicine, Government General Hospital, Madras for their interest and encouragement during the study.

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## Characterization of a toxin from *Parthenium hysterophorus* and its mode of excretion in animals

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**Abstract.** Fractionation of methanolic extracts of air dried aerial parts of *Parthenium* resulted in the isolation of a toxic constituent which was identified as parthenin, the major sesquiterpene lactone from the weed. The LD<sub>50</sub> (minimal lethal dose required to cause 50 % mortality) for parthenin in rats was 42 mg/kg body weight. When [<sup>3</sup>H]-parthenin was given orally or by intravenous administration, radioactivity appeared in the milk of lactating laboratory and dairy animals. Tissue distribution of radioactivity revealed that maximum label was detectable in kidneys.

**Keywords.** Compositae; parthenin; sesquiterpene lactone; LD<sub>50</sub>; toxicity; organelle drug distribution.

### Introduction

Several species of the compositae family are known to be toxic when consumed by animals (Rodriguez *et al.*, 1976; Narasimhan *et al.*, 1977). Incorporation of *Parthenium hysterophorus* into the diet of live stock was found to cause chronic or acute toxicity depending upon the quantum of the weed ingested (Narasimhan *et al.*, 1980). Since no information is available in literature on the chemical constituents of *P. hysterophorus* responsible for vertebrate poisoning, studies were undertaken on the characterization of the toxic principle from the weed and fate of the administered toxin in animals. The results of these studies are presented in this communication.

### Materials and methods

#### Materials

Diphenylamine and calf thymus DNA were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. An authentic sample of parthenin was a gift from Dr. Eloy Rodriguez, University of California, Irvine, California, USA. Parthenin was custom

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Abbreviations used: TLC, Thin layer chromatography; NMR, nuclear magnetic resonance.

labelled with tritium at Bhabha Atomic Research Center, Bombay. All other reagents and solvents used were of analytical grade available commercially.

### *Animals*

Guinea pigs and rats were supplied by the Central Animal Facility of the Indian Institute of Science, Bangalore. A lactating cow which was on an approved nutritional regimen (Maynard *et al.*, 1978) was made available for the experiment by a livestock farm.

### *Isolation of the toxin*

Five hundred g of air dried aerial parts of *P. hysterophorus* were ground into a fine powder and extracted continuously with 1.5 litres of methanol in a Soxhlet's apparatus for 12 h. The resulting dark brown extract was evaporated to dryness in a flash evaporator at room temperature and the residue was designated as Fraction I. This fraction was continuously refluxed with petroleum ether which constituted Fraction II. The residue from Fraction I was further extracted successively with chloroform and ethanol to obtain Fraction III and Fraction IV, respectively. Fraction III was evaporated to dryness, dissolved in hot ethanol and the volume was made up to 200 ml. After cooling, equal volumes of 4% aqueous lead acetate solution was added to this ethanol extract, allowed the mixture to stand at room temperature for 20 min and the resultant precipitate was removed by filtration through a pad of diatomaceous earth. The clear filtrate was concentrated to half its volume to remove ethanol. The aqueous solution thus obtained was extracted with 50 ml portions of chloroform. The pooled chloroform extract was dehydrated with anhydrous sodium sulphate and evaporated to dryness *in vacuo* to obtain Fraction V. The residue was dissolved in 15 ml of a solvent consisting of benzene and acetone (2:1) and subjected to silica gel column chromatography. The column (3 × 55 cms) was equilibrated with benzene and loaded with Fraction V. After washing with 200 ml of benzene, the column was eluted with 200 ml portions of a solvent containing increasing amounts of acetone in benzene (10–100%). The fractions (200 ml each) were evaporated to dryness and analysed by thin layer chromatography (TLC) on silica gel-G plates using benzene:acetone (4:1) or chloroform:diethylether (5:1) as solvent system. The compounds were located on TLC plates by exposing to iodine vapours or by spraying with 5% aqueous potassium permanganate. The fractions yielded, one major and a few minor compounds which were detectable on TLC plates. The fractions that contained the major compound were pooled and subjected to preparatory TLC using benzene:acetone (4:1) as the solvent system. The area corresponding to the major compound on individual TLC plates was scraped and eluted with chloroform. The pooled chloroform eluate was evaporated to dryness and the residue dissolved in a minimal amount of ethyl acetate. The compound was twice crystallized by dropwise addition of cyclohexane in cold to obtain the purified toxin (Fraction VI).

### *Assessment of LD<sub>50</sub> of different fractions*

In order to assess the lethality of different fractions obtained during purification of the toxic principle(s) from *P. hysterophorus*, 10 rats were used for each fraction. Different

doses of each fraction, ranging from 100 to 1000 mg/kg body wt. were administered intraperitoneally. Number of surviving animals in each group was recorded over a 7-day period. Animals that did not receive any test substance served as controls. The dose which brought about 50% mortality ( $LD_{50}$ ) was determined by the method of Litchfield and Wilcoxin (1949) as well as by the probit method of Finney (1952).

#### *Distribution of administered parthenin*

**Excretion and tissue distribution:** One lactating cow and three guinea pigs respectively, were administered intravenously or intracardially with [ $^3H$ ]-parthenin (specific activity 4.9 Ci/mmol: 500  $\mu$ Ci/kg body wt.). Samples of blood, milk, urine and faeces were collected at different intervals (0–72 h). To aliquots (10–50  $\mu$ l) of samples other than faeces, 10 ml of scintillation fluid (methoxyethanol: toluene (1:1) containing 0.5% PPO and 0.05% POPOP) were added and the radioactivity was determined in a LKB, Rackbeta counter. Faecal samples (0.1–0.5 g) were homogenized in 3 ml water and passed through a 100 mesh sieve. Aliquots of the suspension (50 or 100  $\mu$ l) were used to measure the radioactivity.

To follow the distribution of parthenin after oral administration, [ $^3H$ ]-parthenin (1 mCi/kg body wt.) was given orally through an infant tube to 3 lactating guinea pigs and distribution of the label was followed as described above.

After 72 h, the animals were sacrificed by exsanguination and different organs were collected. Tissue samples (0.5–1.0 g) were homogenized in 2–5 volumes of 0.15 M NaCl. An aliquot (50–200  $\mu$ l) of the homogenate was used for the determination of radioactivity.

**Subcellular distribution:** To elucidate the distribution of [ $^3H$ ]-parthenin into subcellular fractions, 3 rats were administered intracardially with [ $^3H$ ]-parthenin (500  $\mu$ Ci/kg body wt.) in phosphate buffered saline (pH 7.4). After 48 h, the animals were sacrificed by cervical dislocation, liver and kidney were collected. Mitochondrial, microsomal and cytosolic fractions were isolated by differential centrifugation (Hogeboom and Schneider, 1950; Johnson and Lardy, 1967). Nuclei were isolated as described by Wang (1967). Protein in samples was quantitated by Biuret method (Gornall *et al.*, 1949) and DNA was estimated by the procedure outlined by Burton (1956). Aliquots of each fraction (100–200  $\mu$ l) were used for the determination of radioactivity.

## Results

### *Purification of toxin from Parthenium hysterophorus*

The toxicity of different fractions obtained during purification of the toxic principle from *Parthenium* weed is presented in table 1. Animals which received only water (controls) or those administered with Fraction II (petroleum ether) or Fraction IV (ethanol fraction) revealed no mortality when observed over a period of seven days. Fractions I and III (methanolic and chloroform fractions) on the other hand, were lethal to rats. Fifty per cent mortality was observed with these fractions at doses ranging from 200–300 mg/kg body wt.

Table 1. Toxicity of various fractions obtained from *P. hysterophorus* to rats\*

Fraction	Intraperitoneally administered dose (mg/kg body wt.)					
	100	200	300	400	500	600
	Animals died/Tested					
I	1/10	2/10	5/10	7/10	9/10	10/10
II	0/10	0/10	0/10	0/10	0/10	0/10
III	0/10	2/10	5/10	8/10	10/10	10/10
IV	0/10	0/10	0/10	0/10	0/10	0/10
V	8/10	NT	NT	NT	NT	NT
VI	10/10	NT	NT	NT	NT	NT

NT: Not tested.

\* Ten per cent stock solutions of Fractions I, II, III, IV and 2% stock solutions of Fractions V and VI were prepared in ethanol. Aliquots of the stock solutions were diluted with water to obtain the desired doses for intraperitoneal administration. The total volume administered was 2 ml. None of the control animals (10 numbers) which were injected with equal volume of water and ethanol died during the experimental period which extended for 7-days.

As shown in table 2, LD<sub>50</sub> analysis of different fractions by the method of Litchfield and Wilcoxin (1949) yielded two dose limits viz., one on the higher side and the other on lower side (330 and 273; 302 and 274; 60 and 48; 42 and 32 mg/kg body wt., respectively, for Fractions I, III, V and VI). Probit method of Finney (1962) yielded LD<sub>50</sub> values of 313, 278 and 51 and 47 mg/kg body wt., respectively with Fractions I, III, V and VI.

Table 2. LD<sub>50</sub> assessment of different fractions from *P. hysterophorus* in rats\*.

Fractions	Litchfield and Wilcoxin Method		Finney's Method
	Lower Limit	Upper Limit	
I	272-73	330-00	313
III	274-29	302-40	278
V	48-29	59-50	51
VI	31-46	42-34	47

\* Dose—mg/kg body wt.

Route of administration—intraperitoneal

20 rats were used to test the lethality of the above fractions at different concentrations. (5 to 500 mg/kg body wt.)

### Identification of toxin

The purified toxin (Fraction VI) was identified as the sesquiterpene lactone parthenin (figure 1) by comparing its properties with those of an authentic sample. Both isolated toxin and parthenin melted at 166°C and had ultra-violet absorption maxima at 215

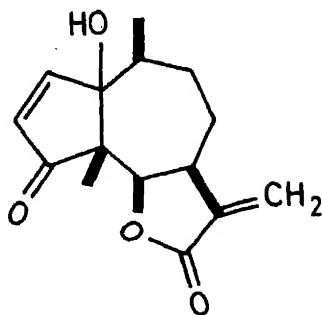


Figure 1. Structure of parthenin.

and 340 nm. The infra-red bands were at 3450, 1755, 1408, 1592 and 1655  $\text{cm}^{-1}$ . The nuclear magnetic resonance (NMR) spectrum of the isolated toxin (parthenin) in  $\text{CDCl}_3$  is shown in figure 2.  $^1\text{H}$  NMR (270 MHz,  $\text{CDCl}_3$  with tetramethyl silane): 7.50 (d,  $\text{H}_2$ ), 6.25 (d,  $\text{H}_3$ ), 6.33 (d, H-13b), 5.61 (d, H-13a), 5.02 (d, H-6), 1.35 (d, C-10 Me). These values correspond to those reported for parthenin by Herz *et al.* (1962).

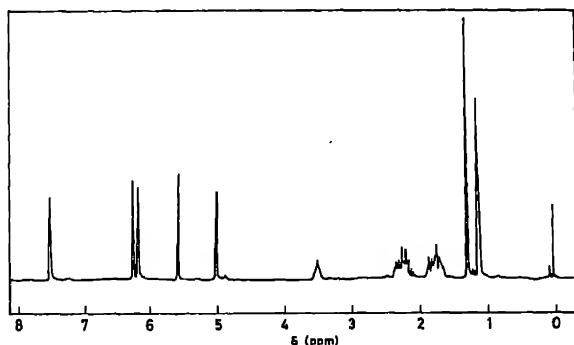


Figure 2. 270 MHz [ $^1\text{H}$ ]-spectrum of parthenin in  $\text{CDCl}_3$ .

#### *Distribution and excretion of parthenin*

**Excretion in urine:** The time course of excretion of [ $^3\text{H}$ ]-parthenin after administration to lactating guinea pigs and a cow by parenteral or oral route, is shown in figure 3. It is apparent that administration of parthenin intracardially to guinea pigs and intravenously to a cow resulted in its excretion in urine within 30 min. Urinary excretion of orally administered parthenin by guinea pigs reached a maximum after 5 h and later declined.

**Excretion in faeces:** No significant amount of radio-label from intravenously administered [ $^3\text{H}$ ]-parthenin was detectable in the faeces of cow. Marginal amount of radio-activity could be detected in faecal samples collected after 5–7 h from guinea pigs injected intravenously with [ $^3\text{H}$ ]-parthenin. Oral administration, however, resulted in excretion of the label in the faeces of guinea pigs upto 72 h with the maximum around 7 h (figure 4).



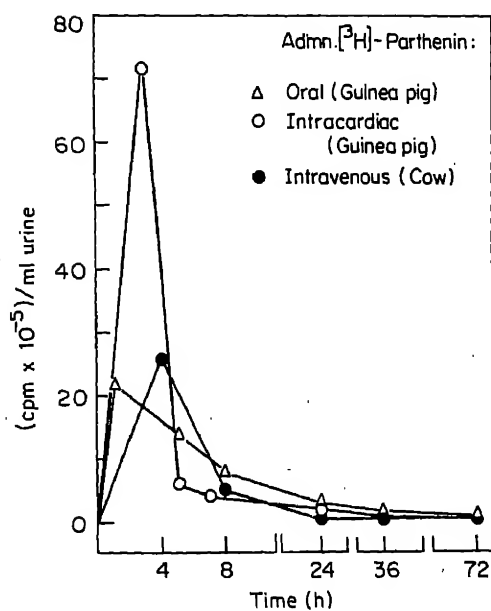


Figure 3. Urinary excretion of  $[^3\text{H}]$ -parthenin in experimental animals.

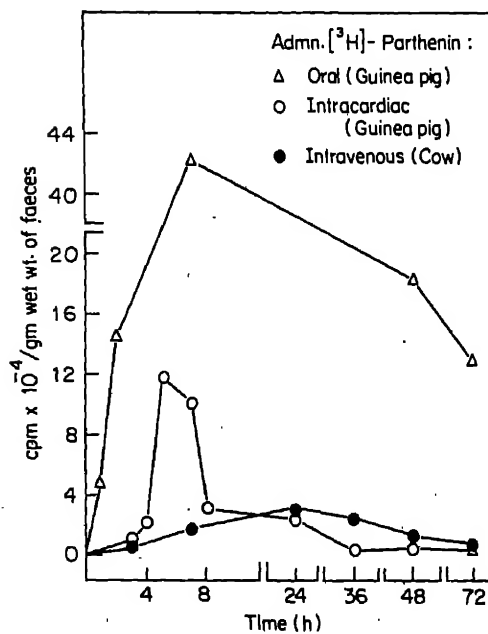


Figure 4. Excretion of  $[^3\text{H}]$ -parthenin in faeces of experimental animals.

**Excretion in milk:** Administration of labelled parthenin intracardially to guinea pigs resulted in the detection of radioactivity in milk as early as 1 h, while in a lactating cow maximum radioactivity was detectable after 5 h. Oral administration of the labelled compound to guinea pigs resulted in the detection of radioactivity in milk after 1 h and the label continued to be excreted even after 72 h (figure 5).

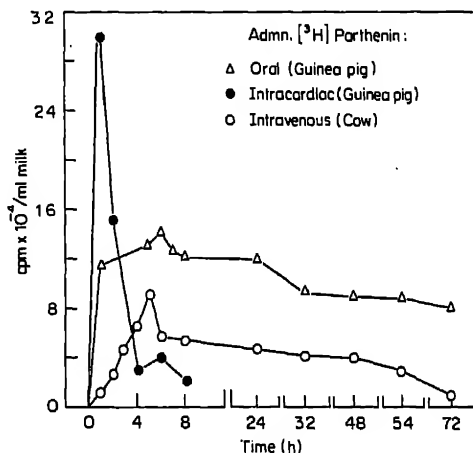


Figure 5. Excretion of [<sup>3</sup>H]-parthenin into the milk of experimental animals.

**Appearance of [<sup>3</sup>H]-parthenin in blood:** The appearance of [<sup>3</sup>H]-parthenin in the blood of guinea pigs administered orally with the labelled sesquiterpene lactone was detectable at the end of second hour. The maximum radio label, however, could be measured after 8 h followed by a gradual decline (figure 6).

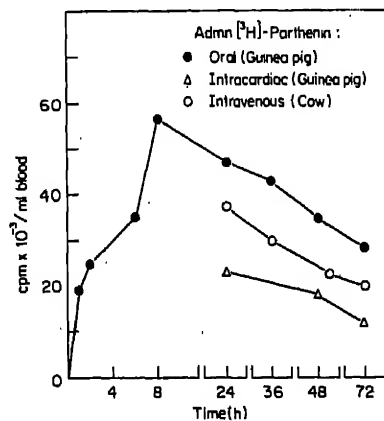


Figure 6. Blood levels of [<sup>3</sup>H]-parthenin in experimental animals.

*Tissue distribution of [ $^3\text{H}$ ]-parthenin:* Measurement of radioactivity in samples of tissues collected from guinea pigs and a cow administered orally or parenterally with [ $^3\text{H}$ ]-parthenin after 72 h revealed that maximum radioactivity was detectable in kidney and liver (table 3). Similarly, bile collected at this time also had considerable radioactivity. The active mammary tissue too, incorporated the label in all the animals (12,000–16,000 cpm/mg protein). A comparison of the distribution of radioactivity in different internal organs revealed that spleen contained the least amount.

**Table 3.** Distribution of [ $^3\text{H}$ ]-parthenin in different organs.

Tissue	cpm/mg protein		
	Guinea pig <sup>+</sup>	Guinea pig <sup>++</sup>	Cow <sup>+++</sup>
Liver	12200	15230	18880
Kidney	24950	38870	74200
Heart	2336	18870	10200
Spleen	1092	1020	1070
Bone marrow	13165	13870	13900
Mammary gland	12240	13410	16340
Skeletal muscle	4959	7660	6700
Lungs	1764	2031	3888
Ovaries	2601	2583	4880
Intestines	9650	8914	9570
Bile†	3094	3701	3816

† cpm/ml

Route of administration: <sup>+</sup> Oral; <sup>++</sup> Intracardiac;

<sup>+++</sup> Intravenous.

From table 4 it is apparent that mitochondrial fractions obtained from both kidney and liver of rats administered intra-cordially with [ $^3\text{H}$ ]-parthenin had highest amount of radioactivity. Microsomal and cytosolic fractions of rat liver contained almost similar quantities of the label, while similar fractions prepared from kidneys had higher radioactivity. The per cent distribution of radioactive label in the kidney and liver of rats administered with [ $^3\text{H}$ ]-parthenin by intracardiac route was 9.4 and 6.6, respectively.

**Table 4.** Subcellular distribution of [ $^3\text{H}$ ]-parthenin in rat liver and kidney.

Organelle	cpm/mg protein	
	Liver	Kidney
Mitochondria	14673 ± 1421	22469 ± 387
Microsome	4941 ± 388	8584 ± 479
Cytosol	4852 ± 112	21532 ± 432
Nuclei*	43648 ± 2362	53426 ± 2119

\* cpm/mg DNA

## Discussion

Fractionation of crude methanolic extracts of *P. hysterophorus* and the evaluation of the isolated fractions for their toxicity in rats, resulted in the identification of parthenin, the major sesquiterpene lactone from the weed as the toxin (figure 1). Although parthenin constituted the major component of the terpene fraction (Fraction V), the toxicity of the minor sesquiterpene constituents of this fraction was not evaluated. However, the increase in the toxicity of parthenin (Fraction VI; LD<sub>50</sub> 42.3 mg/kg body wt. (table 2) over the terpene fraction (Fraction V; LD<sub>50</sub> 59.5 mg/kg body wt.) which contained 75% as parthenin, seems to rule out the possibility of the presence in *P. hysterophorus* of toxic constituents other than parthenin.

The toxicity due to parthenin observed in the present study supports the earlier findings that sesquiterpene lactones contribute to the toxicity of several poisonous species of Compositae to livestock. Witzel *et al.* (1976) isolated a sesquiterpene lactone, helenalin from *Helinium microcephalum* and demonstrated its toxicity to sheep, mice, rats, rabbits and hamsters. Ivie *et al.* (1975a, b) and Kim *et al.* (1975) have also reported that hymenovin, hymenoxan and tenulin were toxic to animals.

The LD<sub>50</sub> values for different sesquiterpene lactones range between 3 and 150 mg/kg body wt. (Ivie *et al.*, 1975a; Kim *et al.*, 1975; Kim, 1980). From the results of the present study it is apparent that the LD<sub>50</sub> for parthenin was 42.3 mg/kg body wt. (table 2).

Hill and his coworkers (Hill, 1977, Hill *et al.*, 1980) observed that oral or intravenous administration of [<sup>3</sup>H]-hymenoxan to rabbits resulted in maximum excretion of the label through urine and bile. Biological half life of the orally administered sesquiterpene lactone (hymenoxan) in blood was estimated to be 9 h. Parthenin also had a similar biological half life in blood (8 h, figure 6).

The rapid excretion of [<sup>3</sup>H]-parthenin in the urine of all the experimental animals supports the earlier observation of Hill (1977) on the pattern of urinary excretion of hymenoxan in animals. He has also observed that accumulation of the toxin was maximal in kidneys. In the present study also, label from [<sup>3</sup>H]-parthenin after administration was concentrated more in kidney and liver than in other organs (table 3). Presence of higher amounts of parthenin in these vital organs substantiates the degenerative changes in liver and kidney of *Parthenium*-fed animals observed by histopathological studies (Narasimhan *et al.*, 1980). Ishida *et al.* (1980) reported that sesquiterpene lactones were excreted as biotransformed glucuronides in both urine and bile of experimental animals. In the present study, however, no attempt was made to follow the metabolic fate of the administered parthenin.

Oral or intravenous administration of [<sup>3</sup>H]-parthenin resulted in the detection of radioactivity in the milk of guinea pigs as early as 1 h, while in the cow, the excretion of the radio label into milk was observed 2 h after oral administration. The excretion of sesquiterpene lactones in the milk of cattle is well documented (Chopra *et al.*, 1965; Dupont and Adda, 1978). The current studies with [<sup>3</sup>H]-parthenin do not exclude the possibility of excretion of parthenin into the milk of dairy animals that occasionally feed on *Parthenium* weed.

It is well established that parthenin is the contact allergen responsible for *Parthenium* dermatitis in humans (Lonkar *et al.*, 1974; Subba Rao *et al.*, 1978). It has been experimentally shown that in buffaloes, dermatitis could be induced by oral administra-

tion of aqueous extracts of *Parthenium* and these animals, like humans, elicit delayed hypersensitive reaction to parthenin (Subba Rao et al., 1979). The results presented in this paper established that parthenin, the major sesquiterpene lactone from *P. hysterophorus* known to be a contact allergen to humans is also a potent toxin responsible for the manifestation of *Partheniosis* (Narasimhan et al., 1980) in livestock fed on the weed.

### Acknowledgements

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## Chemical synthesis, cloning and expression of human preproinsulin gene

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**Abstract.** A 355 base pair DNA sequence coding for human preproinsulin has been assembled by joining 55 synthetic deoxyoligonucleotide fragments prepared by the modified phosphotriester methodology. Proinsulin was expressed under *lac* promoter control and truncated  $\beta$ -galactosidase 590 amino acid long sequence. The fused  $\beta$ -galactosidase proinsulin protein was produced in amount to 30% of the total *Escherichia coli* proteins. It was also expressed in M13 bacteriophage and yeast system.

**Keywords.** Synthetic DNA; recombinant DNA; plasmid DNA; *lac* promoter;  $\beta$ -galactosidase.

### Introduction

Since the discovery of insulin in 1922 by Banting and Best, many millions of lives have been saved. The therapeutic approach to diabetes has rapidly progressed over wide areas of basic chemical and biological science. Advances during the last decade have resulted in the maturation of rapid methodology of DNA synthesis, sophistication in recombinant DNA techniques and rapid DNA sequence method. It seems safer to predict that during eighties large-scale and economical synthesis of important proteins, *e.g.* insulin, human growth, interferons and vaccines, etc. directed by genes will be possible. Since 1973 our laboratory has been actively involved in the development of modified phosphotriester methodology of oligonucleotide synthesis and their application in refining recombinant DNA technology such as use of linkers, adaptors, retrieval adaptor, primers for DNA sequence and DNA probing. In this paper we wish to review our work towards the total synthesis of 355 b.p. DNA sequence coding for human preproinsulin and studies on its expression in *Escherichia coli* and yeasts.

### *The chemistry of insulin*

The gene for preproinsulin is transcribed in the islet cells of the endocrine pancreas into a messenger RNA (mRNA) precursor, which is then spliced to produce mature mRNA. This mRNA is translated into preproinsulin, which is sequentially processed into proinsulin and finally into insulin. The general structure of a preproinsulin molecule is

NH<sub>2</sub>-preregion-(B chain)-C chain-A chain-COOH. The first process is the proteolytic elimination of the preregion which serves as a hydrophobic signal for the transport of the nascent chain through the microsomal membranes of the endoplasmic reticulum. In proinsulin the two regions of the polypeptide chain of mature insulin, the B and A chains, are connected by a middle peptide, the C peptide, flanked by two pairs of basic amino acids, Arg-Arg and lys-Arg, figure 1. In the last stage of maturation, proteolytic enzymes cleave of the basic amino acids to release C peptide and form mature insulin where B chain 30 amino acids and A chain 21 amino acids are joined by two disulphide cross-linkages.

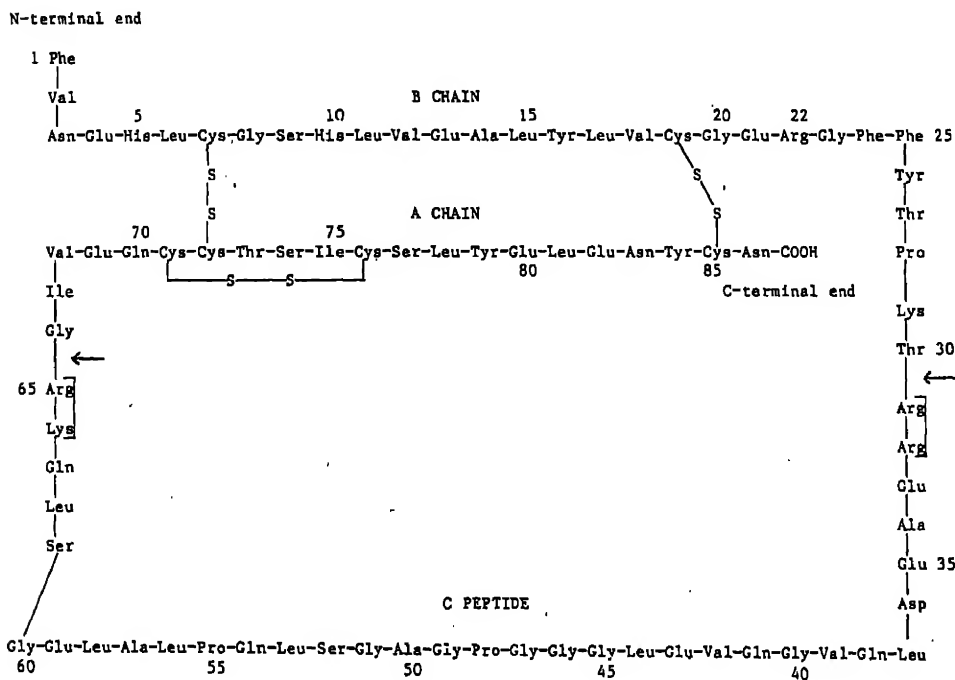


Figure 1. Amino acids sequence of human proinsulin.

### *The structure of human preproinsulin genes*

Preproinsulin gene of the human has been isolated from the chromosomal DNA library using homologous or heterologous cloned cDNA. The coding region of the corresponding mRNA including the initiation and termination codons consists of 333 nucleotides. Seventeen nucleotides upstream from the AUG initiator, the segment corresponding to the 5'-noncoding region of the mRNA, is interrupted by an intron of 179 b.p. long. A second large intron of 786 b.p. occurs between the sixth and seventh amino acid of C peptide.

## Rationale for DNA Synthesis

Among the several possible ways in which an eukaryotic gene can be obtained and expressed in prokaryotes, chemical synthesis offers the following advantages: (i) it gives directly the exact desired sequence, the coding sequence and the non-coding sequences can be designed at will for prokaryotic expression, restriction sites can be removed or built in, introns deleted; (ii) it bypasses the often difficult step of isolating the relevant mRNA or genomic DNA; and (iii) it simplifies the modification of the gene and its product protein by lengthening or shortening the coding region, or by changing specific codons and the corresponding amino acids.

The human proinsulin DNA sequence assembled in our laboratory was derived from the amino acid sequence (Oyer *et al.*, 1971) by using the genetic code, and guided by the rat proinsulin DNA sequence (Ullrich *et al.*, 1977) (figure 2). The human proinsulin shares 95 %, 90 % and 71 % amino acid sequence homology for the A, B, and C chain, respectively, with the rat proinsulin. Wherever the amino acid sequence is the same between the two species, the rat proinsulin nucleotide codon was chosen for the synthetic human proinsulin coding sequence. In addition, we also synthesized a modified pre-sequence of human proinsulin (Goeddel *et al.*, 1980). The present sequence contains four substitutions at positions 2-alanine replaced with glycine, 5-methionine with isoleucine, 11-leucine with isoleucine and 15-alanine with isoleucine. These changes were introduced to increase restriction sites, such as four *Sau*3A and two *Bam*HI sites in the codon region which will allow further modification of the pre sequence for detailed study. Moreover, these substitutions at four different positions do not alter the hydrophobic property of the pre sequence.

## Chemical synthesis

The fundamental objective in oligonucleotide synthesis is the formation of an ester linkage between an activated phosphoric acid function of one nucleotide with the hydroxyl group of another nucleoside/nucleotide, thus ultimately forming the natural phosphodiester bridge between the 5'-OH of one nucleoside unit and the 3'-OH of the next. To achieve these ends, the nucleic acid chemist must devise (i) selective blocking/deblocking procedures for a primary, and a secondary OH (or two in ribonucleosides), a primary amino group and often two of the three dissociable groups of phosphate. In addition he must be cognizant of the variable labilities of certain purines and pyrimidines and their glycosidic bonds plus the variable reactivities of mono-, di- and trisubstituted phosphate. All protecting groups must be removed without causing chain cleavages and with minimal formation of side products. The required product must be separated from these almost identical impurities and methods devised to prove unambiguously the structure.

Until 1974, all of the oligonucleotides of biological significance were synthesized chemically by the phosphodiester method as pioneered by Gilham and Khorana (1958). However, there were inherent difficulties in this method such as low yields with increasing chain length caused by unprotected internucleotidic phosphodiester linkages and time-consuming purification procedures.

A possible solution to some of these problems (water solubility, ion exchange





chromatography and lower yields) inherent in the phosphodiester approach would accrue if the third dissociation of phosphate was marked, thus creating a neutral organic molecule amenable to the more standard manipulations of organic chemistry.

The basic principle of the triester method is to mask each internucleotidic phosphodiester function by a suitable protecting group during the course of building a defined sequence. After completing synthesis, all the protecting groups are removed at the final step to give a deoxyoligonucleotide containing each internucleotidic 3' → 5' phosphodiester linkage. The main advantages of this method include the opportunity for large-scale (50–75 g) synthesis, significantly shorter time periods especially in the purification steps and high yields using almost stoichiometric amounts of reactants. This is probably due to the absence of any *endo*-P-O- groups in the oligonucleotide chain thus avoiding chain scission and pyrophosphate formation.

The triester method as originally reported (Letsinger and Mahadevan, 1965; Reese and Saffhill, 1968; Eckstein and Risk, 1969) involved the phosphorylation of the 3'-OH group of a 5'-protected nucleoside with a substituted phosphate followed by subsequent condensation with a primary 5'-OH of a second nucleoside. This is essentially a one-pot procedure. However, it was observed that owing to incomplete phosphorylation in the first state, subsequent coupling with 5'-protected nucleoside led to a complicated reaction mixture including the so-called 3'-3' and 5'-5' coupled products. Since these mixtures could not be completely resolved on conventional silica gel columns, the advantages of large-scale synthesis and high yield of product were somewhat nullified.

#### *Modified phosphotriester approach*

To overcome this difficulty the one-pot synthetic approach which includes phosphorylation and coupling steps was modified to a two-step sequential procedure (Narang *et al.*, 1979). The basic feature of this approach is to start the synthesis of an oligonucleotide from a totally protected mononucleotide containing a fully masked 3'-phosphotriester group (figure 3). Since the resulting intermediate oligonucleotides contain a fully masked 3'-phosphate, the necessity for a phosphorylation step at each condensation stage is eliminated thus simplifying the approach.

Such a starting material was prepared by treating 5'-dimethoxytrityl N-acyl deoxymononucleoside with *p*-chlorophenyl phosphoryl ditriazolides followed by cyanoethylation reaction. The fully protected monomer is easily purified and permits chain elongation from either end. At each coupling step the  $\beta$ -cyanoethyl group was removed selectively on treatment with triethylamine-anhydrous pyridine or diisopropylamine-anhydrous pyridine. Under these conditions all the other base labile (N-acyl and *p*-chlorophenyl) protecting groups were intact. In practice, longer-sized chains are built by block additions starting from the dinucleotide unit. After each cycle the desired fully protected product can be purified on a short column, medium and high pressure techniques, reversed phase technique on RP-2 and a simple but very effective chromatography on deactivated silica gel with an aqueous solvent system such as acetone-water ethyl acetate. The complete deblocking was done first by acid treatment, followed by concentrated ammonia, and the desired product was purified by preparative electrophoresis on 15% acrylamide. Each of the fragments was checked for its base sequence by the mobility method (Tu and Wu, 1979).

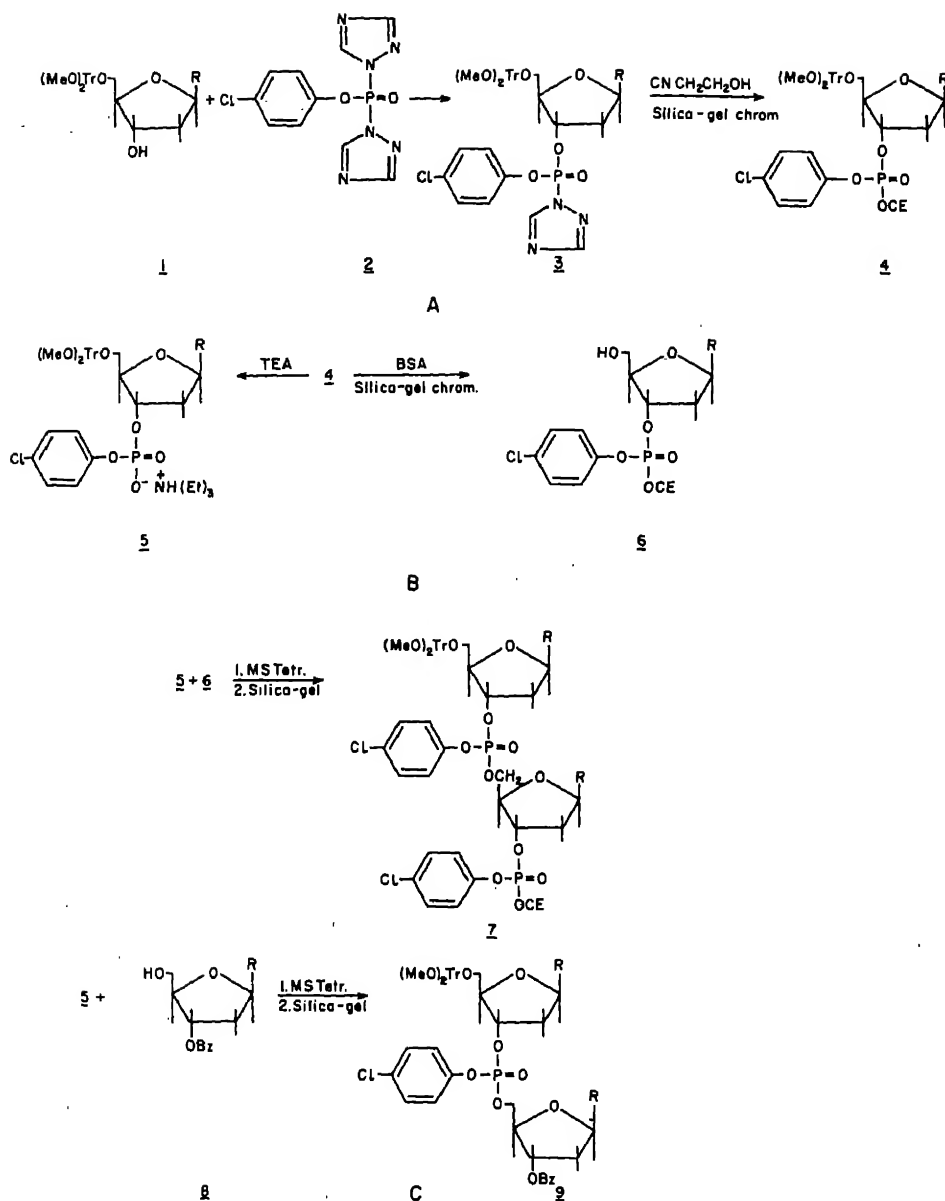


Figure 3. Modified phosphotriester method.

## Enzymatic assembly

### Synthesis of double-stranded DNA

Once the short segments comprising the DNA sequence of both the strands are synthesized, the next task is aligning them in proper order and linking them covalently

by DNA ligase enzyme. A minimum of four overlapping nucleotide pairs are required on each side of the junction to provide sufficient template interaction for the joining of deoxyribonucleotides with DNA ligase enzyme.

An alternative to this approach is the synthesis of a partial strand (upper and lower) by DNA ligase reaction. The resulting single strands are purified by gel electrophoresis under denaturing conditions. Since the 3'-end of the upper strand is complementary to the 3'-end of the lower strand by an overlapping stretch of nucleotide, a partial duplex is formed. With the single strand region as template the remainder of the duplex was enzymatically synthesized with Avian Myeloblastosis virus reverse transcriptase and the four deoxynucleoside triphosphates to yield a complete duplex DNA. Using the approach a 63 bases long duplex for human insulin gene A was synthesized and confirmed by its DNA sequence method.

#### *Building the B- and C-chain assemblies and its cloning*

The subassemblies each containing either 4 to 6 chemically synthesized fragments, were assembled in the conventional manner (Brown *et al.*, 1979) using T4 DNA ligase. To insure purity of the finished subassemblies, each ligation was performed using only 3 fragments at a time, the 2 to be ligated and the complementary fragment aligning them. The ligated oligonucleotide in the reaction mixture was then separated on a denaturing polyacrylamide gel. This made identification of the desired product unambiguous, since only one band of high molecular weight ligation product could be expected on any given gel. Although time consuming, we feel such a procedure ensures the accuracy in the enzymatic synthesis of a long fragment.

After both strands of a subassembly had been completed, they were annealed together by slow cooling from 95°C to room temperature in 0.5 M Tris HCl buffer, pH 7.5. The three resulting double-stranded subassemblies of B-chain were then ligated in a single step while the three of C-chain were ligated in 2 steps.

The phosphorylated BC-chain DNA was then ligated to the *EcoRI*-*Bam*HI large fragment of plasmid pWJ3, which contains the 3986 bp long *EcoRI*-*Bam*HI fragment of pBR322 and a 1776 bp long *EcoRI*-*Bam*HI fragment of yeast DNA. The *Bam*HI and *Sau*3AI sites have identical cohesive ends and thus can be ligated. The ligation mixture was used to transform competent *E. coli* HB101 cells. The resulting 83 transformants were screened with a [<sup>32</sup>P]-labelled B-chain probe made from subassembly SB-1. Three strongly hybridizing colonies were picked for further study. After restriction enzyme digestion, two of these, clones 43 and 68, were found to show the proper size insert: 190 bp by *EcoRI* and *Bam*HI double digestion, and 230 bp by *EcoRI* and *Hha*I double digestion. DNA sequence analysis showed that clone 43 gave the expected sequence, but the GC on position 22 in clone 68 was mutated to an A-T bp possibly during the plasmid replication.

#### *Building the insulin A-chain coding sequence from synthetic oligodeoxynucleotides*

Figure 4 illustrates the general scheme for enzymatic conversion of these segments into a 63-nucleotide duplex encoding the human insulin A-chain (structure C). In the first

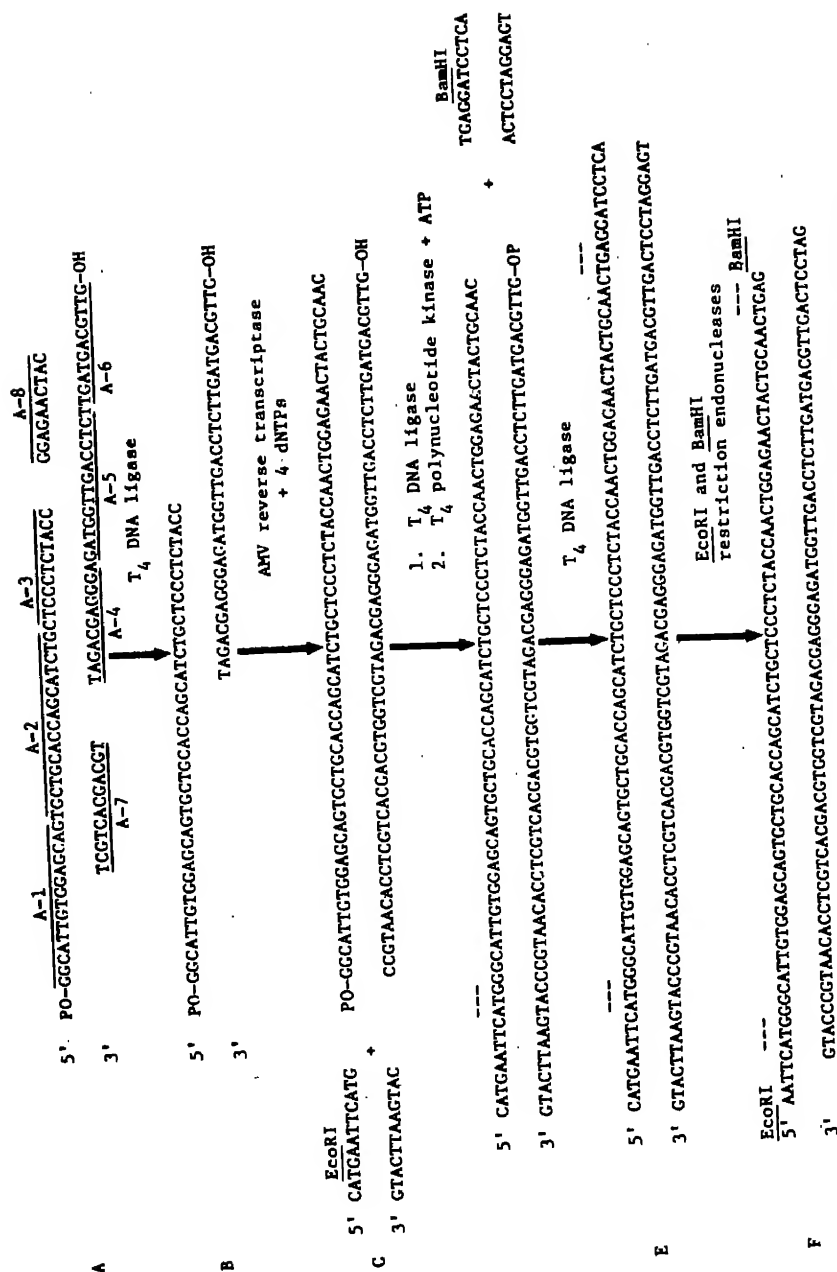


Figure 4. Scheme for assembly of the human insulin A-chain gene from synthetic oligomers.

step, 5'-phosphorylated fragments A-1, A-2 and A-3 were ligated together using fragments A-7 and A-4, complementary to the sequence surrounding the 2 junction points, to provide the duplex structure necessary for ligation. Oligomers A-4, A-5 and A-6 were joined in a similar manner. The resulting 43- and 36- nucleotide-long single strands were purified by gel electrophoresis under denaturing conditions, and they were annealed to form a partial duplex (structure B). With the single-stranded regions used as templates the remainder of the duplex was enzymatically synthesized with Avian Myeloblastosis virus reverse transcriptase and the 4 deoxynucleoside triphosphates (Scarpulla *et al.*, 1982). Fragment A-6 was not phosphorylated so that the *EcoRI* start adaptor shown in figure 5 (line C) would most efficiently ligate to its correct position at the 5'-end of the gene. After purification of the start signal ligation product, the free 5'-OH was phosphorylated and the *BamHI* stop signal adaptor ligated to the right-hand end of the duplex.

The completed insulin A-chain gene with the start and stop signals was cleaved with *EcoRI* and *BamHI* to give the required sticky ends (structure F) and the fragment was cloned into the plasmic vector pBR322 and used to transform *E. coli* strain M94 (B. Bachmann, *E. coli* Genetic Stock Center). From an estimated 0.2 pmol aliquot of synthetic A-chain gene, 114 transformants were isolated. Colony hybridization using a specific [<sup>32</sup>P]-labelled probe generated from repair synthesis of the A-chain 36-mer template with fragment A-8 acting as primer, revealed that 22 of 114 tetracycline-sensitive transformants contained insulin sequences. The result of sequence analysis of one of these clones, designated pSIA12 (SIA: synthetic insulin A-chain gene) establishes the correct sequence of the cloned human insulin A-chain gene expected from the synthetic scheme.

*Use of a chemically synthesized Mbo II retrieval adaptor to regenerate the blunt-ended insulin A-chain duplex*

To use pSIA12 as a source of insulin A-chain for future work adjoining it to a synthetic insulin C-chain gene, it was of interest to regenerate the 5'-end of the cloned 63-nucleotide coding duplex. After *EcoRI* cleavage, eight nucleotides contributed by the start adaptor needed to be removed. This was accomplished by a new method using a symmetrical adaptor having an *MboII* recognition sequence at its end. The scheme, outlined in figure 5, for removal of the desired number of nucleotides relies on the fact that cleavage with *MboII* occurs eight nucleotides downstream from its recognition site, 5'-GAAGA. After filling in the cohesive end of the *EcoRI* site using reverse transcriptase, the *MboII* adaptor is ligated to the resulting blunt end (structure B). Subsequent digestion with *MboII* removes all but a single protruding 3'-nucleotide (structure D). This was removed by the 3'-exonuclease and the exchange reaction catalyzed by the large fragment of DNA polymerase I. After treatment with DNA polymerase I (large fragment) and dCTP, the blunt end of the fragment was ready for ligation to chemically synthesized complementary C-chain oligomers, C-8 and C-17, which end in a 5'-protruding (GATC) *MboI* (or *Sau3AI*) recognition sequence. The modified insulin A-chain gene was cloned into the *BamHI* site of pBR322 to give pSIA34.

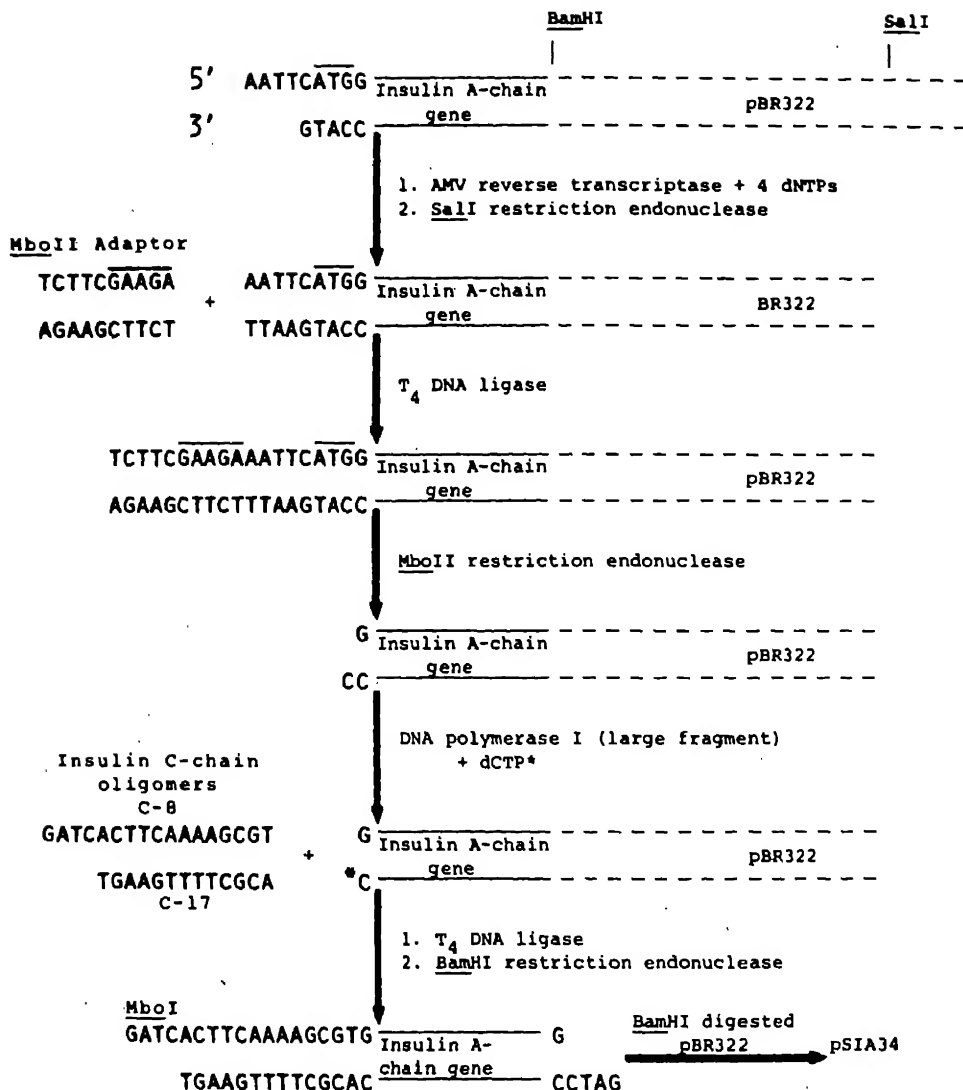


Figure 5. Scheme for generating the blunt-ended human insulin A-chain duplex free of extraneous nucleotides using a synthetic *MboII* retrieval adaptor.

#### Ligation of A-chain DNA to BC-chain DNA in a plasmid

The insulin A-chain assembly was retrieved from the pSIA34 plasmid (Scarpulla *et al.*, 1982) by digestion with *Sau3AI*. Next, plasmid 43 with BC-chain DNA was cut with *BamHI* and ligated to the *Sau3AI* site of the retrieved A-chain DNA (figure 6). Since the latter has identical sticky ends and could insert in either of two orientations and since plasmid 43 could also close back without inserting the A-chain DNA, we used two types of hybridization to screen for the desired transformants. First, we screened 300

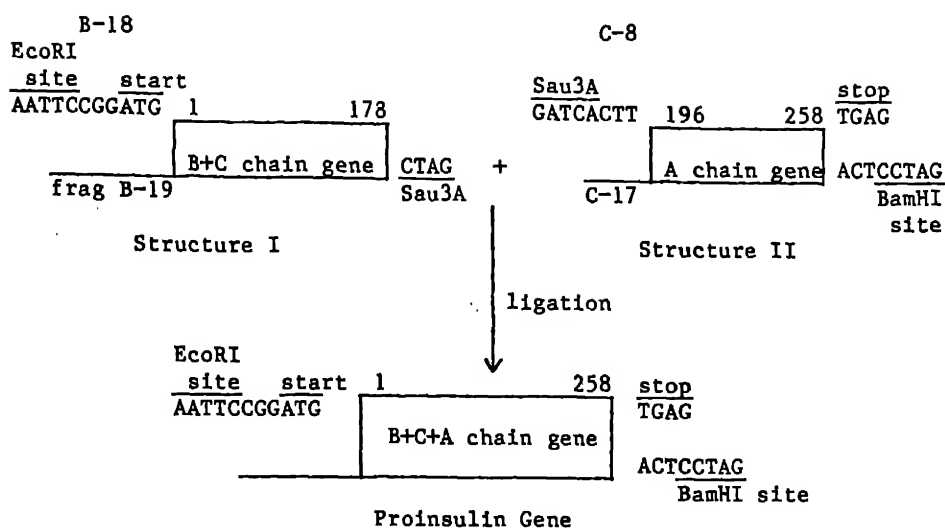


Figure 6. Scheme for joining BC-chain DNA to A-chain DNA in a plasmid.

transformants (picked at random) with a labelled oligonucleotide fragment from the A-chain assembly, fragment C-8, to find those colonies where the plasmids has incorporated A-chain DNA. In this step, 35 transformants were found to hybridize at 12°C in 50% formamide and 5 × SSC. To find out which of these colonies had inserted the A-chain DNA in the proper orientation relative to BC-chain DNA, we constructed a 30-base long orientation probe corresponding to fragments C-17, C-16 and a part of C-15. Under the appropriate hybridization conditions, this probe is expected to bind only to those colonies where fragments C-7 of structure I and C-8 of structure II were both present and continuous. Indeed, of 35 colonies shown to contain A-chain DNA by hybridization to C-8 alone, 16 also hybridized to this orientation probe under more stringent conditions (23°C, 50% formamide, 5 × SSC). Of these 16 colonies, 6 contained a single insert of the appropriate size (277 bp) as shown by *EcoRI*-*BamHI* digestion of the plasmid.

#### Sequencing of the chemically synthesized and cloned proinsulin coding DNA

To characterize the final product, it was sequenced by three different methods. Using the chemical method, the major portion of the BCA-chain DNA was sequenced in both strands and the sequences were found to be complementary as expected. The sequence of nucleotides 40–220 was also confirmed by using the exonuclease III method (Brousseau *et al.*, 1982).

#### Synthesis of pre DNA sequence and its joining to BCA proinsulin

The 72-bp pre sequence flanked by *EcoRI* termini was assembled by stepwise joining of twelve synthetic fragments (LI to L12) in the presence of non-radioactive complementary strand using T4-DNA ligase. This was then joined to *EcoRI*-digested plasmid pSI-BCA4 and the mixture was used to transfect competent cells. Two positive clones were identified by colony hybridization and were recloned in M13mp8 (Georges *et al.*, 1984).



### Site-specific mutagenesis

Since we intended to study the expression of preproinsulin in various systems besides M13 it was desirable to remove the *Eco*RI site between pre region and proinsulin. It was achieved by site-specific mutagenesis deleting an ATT triplet using a synthetic 19-mer primer. Selection of the clones by colony hybridization at various temperatures up to 58°C gave two positive clones, one with a deletion of the AAT triplet from the sequencing strand. This was also confirmed by sizing the *Eco*RI-*Hind*III fragment containing the complete sequence of preproinsulin DNA on a 4% polyacrylamide gel.

### Expression studies

#### *In E. coli* bacteria

The application of recombinant DNA techniques has resulted in the cloning of human genes which can direct the synthesis of large amounts of valuable proteins in microorganisms. Lower molecular weight proteins such as somatostatin, insulin and proinsulin produced in *E. coli* were rapidly degraded unless these proteins were fused to a large *E. coli* enzyme. One commonly used *E. coli* system for making *in vivo* fusion protein with the small proteins of interest is the *lac* system which includes the coding sequence of the first 1007 amino acids of  $\beta$ -galactosidase.

The amino acid sequence of *E. coli*  $\beta$ -galactosidase and the DNA sequence of the  $\beta$ -galactosidase gene have been reported. Since the *lac* system is well defined it is most suitable for further molecular manipulations to improve the system for more efficient expression of foreign proteins. For example, it is of interest to construct several families of plasmids that code for stable truncated  $\beta$ -galactosidase, and to determine the minimum length of a truncated  $\beta$ -galactosidase that can produce a stable fusion protein. For the production of the largest amount of a particular protein, the shorter the truncated  $\beta$ -galactosidase in the fused protein the higher the percentage of the desired protein can be obtained.

We have constructed various expression vectors containing truncated  $\beta$ -galactosidase gene as leaders for expression of a foreign protein in *E. coli*. Poly-linker sequence containing convenient restriction sites has been fused in all three reading frames at the carboxyl termini of the shortened *lacZ* sequences. For studying expression in this system we have used a synthetic human proinsulin gene, and found that up to 30% of the *E. coli* protein is represented by the 590 amino-acid-long truncated  $\beta$ -galactosidase fused to the 86-long proinsulin.

The amount of  $\beta$ -galactosidase fused to proinsulin synthesized in *E. coli* was first estimated by gel electrophoresis. The *E. coli* cells carrying plasmids pSI-1007b, pWR590-BCA4, and pWR450-BCA4 were cultured for 8 h or overnight (18 h). The cells were collected and lysed. Figure 7 presents the separation of the fused polypeptides on a 10% polyacrylamide gel. Samples were prepared by 6-fold dilution of the guanidine-HCl extracts with buffer containing phenylmethylsulfonyl fluoride, the precipitated water-insoluble proteins were washed, dissolved in a sodium dodecyl sulphate-containing sample buffer and electrophoresed. It can be seen that strains harbouring plasmids pWR450-BCA4, pWR590-BCA4 and pSI-1007b produced large amounts of

fused polypeptide, not found in the wild-type strain of *E. coli* (figure 8) by electron microscopy. The molecular weights of these fusion polypeptide products are 59 KDal, 70 KDal and 100 KDal, respectively, and they agree well with those predicted from the DNA sequences (figure 8) (Guo Li-He *et al.*, 1984).

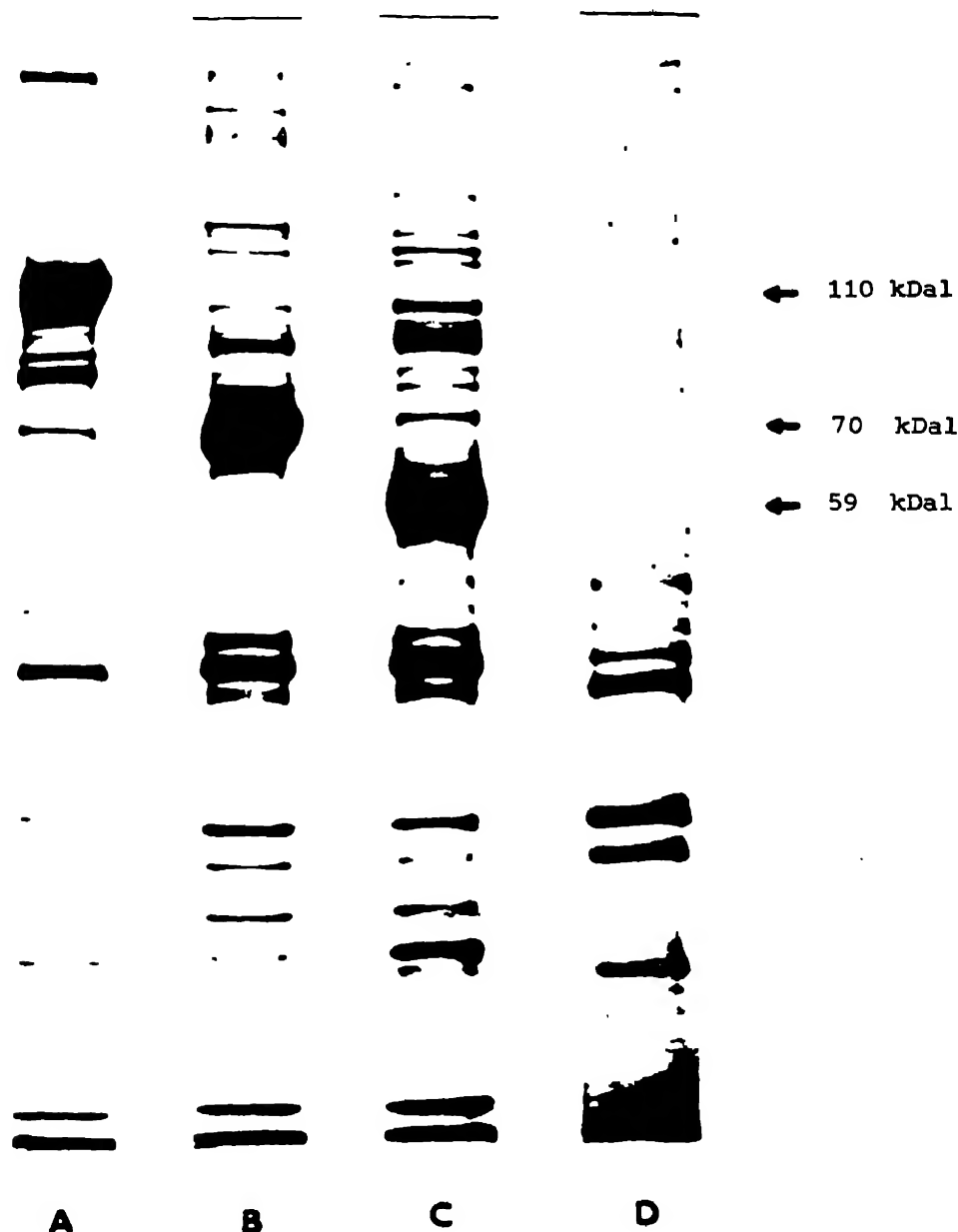


Figure 7. Gel pattern of  $\beta$ -galactosidase-proinsulin fused proteins synthesized in *E. coli*. A. pSI-1007b; B. pWR590-BCA4; C. pWR450-BCA4; D. Control *E. coli* strain 5346.



Figure 8. An electron micrograph of the *E. coli* transformed to produce fused proinsulin protein ( $\times 40,000$ ).

*Radioimmunoassay for proinsulin synthesized in E. coli*

$\beta$ -Galactosidase-proinsulin fused protein synthesized in *E. coli* cells carrying plasmids pSI-1007b, pWR590-BCA4 or pWR450-BCA4 was measured as human C-peptide using radio-immunoassay. In general, the anti-C antibody recognizes the C-chain of proinsulin at an efficiency of approximately 10%, as compared to the isolated C-chain. A correction factor of 10 was applied to the data given in table 1.

Table 1. Expression of proinsulin in *E. coli* 5346. The synthetic human proinsulin gene was cloned into the following plasmids containing different lengths of the  $\beta$ -galactosidase coding sequence.

<i>E. coli</i> strain harbouring plasmid	$\mu\text{g}$ proinsulin/mg <i>E. coli</i> protein		Culture in stationary phase OD <sub>550</sub> = 2
	OD <sub>550</sub> = 0.8		
	non-induced	+ IPTG	
pSI-1007b	1.3	5.0	16.7
pWR590-BCA4	0.9	6.6	41.5
pWR450-BCA4	1.8	3.7	26.7
Control <i>E. coli</i> with no plasmid	0.0	0.0	0.0

Table 1 gives the levels of proinsulin expressed per mg of total cell protein. In the logarithmic phase of growth, both IPTG-induced and non-induced *E. coli* cells synthesize  $\beta$ -galactosidase-proinsulin fusion polypeptides, however, the levels of this synthesis are higher in induced cultures. When the cultures reached stationary phase the levels of expression were significantly greater, indicating accumulation of the fused proteins.

Data presented in table 1 indicate that the length of the leader has a significant influence on the level of proinsulin expression. When no leader polypeptide, or when an 8 amino acid long leader is present, no proinsulin could be detected in *E. coli* (data not shown). The presence of long leaders, consisting of 1007, 590 and 450 amino acids of  $\beta$ -galactosidase in front of the proinsulin gene resulted in very high expression of fusion proteins. Strain pWR590-BCA4 consistently produced more proinsulin per mg of total *E. coli* protein than the others. If the correction factor we used in the quantitative determination of the proinsulin produced in *E. coli* is correct, then 41.5  $\mu\text{g}$  of proinsulin produced in pWR590-BCA4 is equivalent to 326  $\mu\text{g}$  of the fused  $\beta$ -galactosidase-proinsulin. In other words, 32% of the total *E. coli* protein was the fused protein in this experiment.

*Release of proinsulin from the fusion polypeptides by CNBr cleavage:* The synthetic human proinsulin gene was constructed with a methionine codon immediately preceding the first amino acid codon of the mature insulin B-chain. It was possible, therefore, to release proinsulin from the fused polypeptides by cyanogen bromide

cleavage at the methionine residue. The purified proinsulin was efficiently converted to biologically active insulin by digestion with trypsin and carboxypeptidase *B* (Kemmler *et al.*, 1971; Chan *et al.*, 1981).

### M13 Bacteriophage

In our present studies, we have also made use of small single-stranded DNA bacteriophage M13mp 8 vector for cloning, sequencing, site-specific mutagenesis and expression studies of synthetic human preproinsulin gene. M13 vector already has a *lac* promoter and several useful restriction sites in the  $\beta$ -galactosidase gene, and the synthesis of the fused gene product is inducible. The main advantages of this vector are (i) its high copy number of 200–300 molecules per bacterium, (ii) its suitability for site-specific mutagenesis followed by dideoxy sequencing to identify the mutants, (iii) its ability to produce reasonable levels of an eukaryotic protein such as interferon.

In order to simplify isolation and purification of a pure protein from genetically engineered bacteria, we thought of attaching a short stretch of homopolypeptide sequence before the pre sequence. For example, polycysteine, polyglutamine, polylysine, polytryptophane or polyleucine could change the physical property of the desired product so that its isolation from the complex mixture of the cell protein may become easier by affinity chromatography. In this connection we synthesized DNA of 25 bp length to introduce TGT codon of (cysteine)<sub>7</sub> in one strand and CCA codon (glutamine)<sub>7</sub> on the opposite strand preceding the pre sequence. This affinity leader sequence put preproinsulin gene in phase with the  $\beta$ -galactosidase AUG initiation codon. In the case of (glutamine)<sub>7</sub> leader, an expression was observed whereas with seven cysteine leader the expression was undetectable. Whether this was due to rapid degradation caused by the cysteine leader, interference of the cysteine with the immunoassay system or some other reason will be investigated.

### In yeast

The proinsulin gene has been cloned in yeast behind the ADH1 promoter. Under these conditions, however, although abundant transcripts were detected no proinsulin protein was found even by the extremely sensitive C-chain radioimmunoassay. This parallels a similar finding of good transcription of the gene for rat growth hormone in yeast but no detectable protein. In the latter case the polyA mRNA was isolated and translated in a reticulocyte *in vitro* system to yield authentic rat growth hormone. Two possibilities are suggested for this absence of a product, one is that there is some barrier to the translation of this mRNA in yeast, the other possibility is that the foreign protein is rapidly degraded by yeast. To examine the latter possibility we fused the proinsulin gene to the amino portion of the yeast galactokinase (GAL1). Previous work with *E. coli* has shown that small proteins can be formed by fusing them with a  $\beta$ -galactosidase protein leader. We found that a longer galactokinase leader (280 amino acids) gave a better yield of proinsulin than a short (30 amino acids) leader (Stepien *et al.*, 1983).

It would be preferable for production of proinsulin on an industrial scale to have the proinsulin secreted and possibly also processed to insulin by yeast. Yeast has a typical mammalian system for the secretion of proteins and the killer toxin of yeast undergoes

an extensive series of processing events during its secretion. Our initial observations on the expression of the preproinsulin gene in yeast is that when transcribed from the ADH1 promoter, in contrast to the proinsulin gene, we were able to detect both intra- and extracellular proinsulin. Additionally it appears that the extracellular protein had undergone at least one proteolytic cleavage.

### Concluding remarks

The rapid synthesis of tailored-made gene, its cloning and expression in the living system has become a powerful tool to program microorganisms to make proteins that do not occur naturally in any organism. This approach of synthesis could provide a wealth of new molecules when more is known about the relationship between the architecture of proteins and their biological proteins. Ultimately this branch of science may find its best application in solving basic problems such as health, food and energy so essential to mankind.

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## Modified bases in transfer RNA

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**Abstract.** Transfer RNA is uniquely enriched with modified bases. A large body of information has accumulated about the occurrence, nature and distribution of modified bases in tRNA. But similar investigations on the enzymes involved in this post-transcriptional modification have been hampered by the instability of the enzymes and lack of suitable substrates. The present review summarises briefly, the occurrence and methods of detection of modified bases, the enzymes involved in their formation and also certain suggestive evidence for the role of modification in cellular metabolism.

**Keywords.** tRNA; methyltransferases; methylation base modification; function.

### Introduction

Transfer RNA entered the field of molecular biology research in 1958 when Crick proposed the role for an adaptor molecule in the translation of genetic messages. Thereafter the presence of such a molecule was experimentally verified and further it became clear that once the amino acid is attached to the cognate tRNA, it is the tRNA that dictates the translocation of the amino acid in the protein synthesising machinery. Thus tRNA plays a pivotal role in information transfer in the cell.

Over the years, the tRNA molecules by the virtue of their chemical stability unlike mRNA, their small size (70-80 nucleotides long) and their relative abundance turned out to be one of the widely studied and well investigated components of the cell. Now we know the primary structure of more than 180 tRNA molecules from various sources as well as their generalised three dimensional crystal structure at 2.5 Å resolution.

The studies on tRNA have been very fruitful with the development of various methodologies for the purification and separation of single species tRNA (Nishimura, 1971 and references therein). The methods of reversed phase chromatography (Kelmers *et al.*, 1965) and electrophoresis on polyacrylamide gel (Ikemura and Dahlberg, 1973) have made it possible to purify single species of tRNA starting from small quantities of total tRNA.

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Abbreviations used: mcm<sup>5</sup>U, 5-Methoxycarbonyl methyl uridine; cmnm<sup>5</sup>U, 5-carboxy methyl aminomethyl uridine; acp<sup>3</sup>U, 3-(3-amino-3-carboxy propyl) uridine; m<sup>5</sup>C, 5-methylcytosine; ac<sup>4</sup>C, N<sup>4</sup>-acetyl cytidine; s<sup>2</sup>C, 2-thiocytidine; i<sup>6</sup>A, N<sup>6</sup>-isopentenyl adenosine; ms<sup>2</sup>i<sup>6</sup>A, 2-methyl thio-N<sup>6</sup>-isopentenyl adenosine; t<sup>6</sup>A, (N-(9-β-D-ribofuranosyl purin-6 yl) carbamoyl) threonine; m<sup>1</sup>G, 1-methyl guanine; m<sup>2</sup>G, 2-methyl guanine; m<sup>2</sup>G, N<sup>2</sup>N<sup>2</sup>-dimethyl guanine; m<sup>7</sup>G, 7-methyl guanine; NMR, nuclear magnetic resonance; HPLC, high pressure liquid chromatography; AMV, avian myeloblastosis virus.



Hand in hand with this development are the methods developed for sequencing of tRNA. These include various enzymatic as well as chemical methods as a result of which now it is possible to decipher the sequence starting from 5–10  $\mu$ g of pure tRNA species (Nishimura and Kuchino, 1983).

It has also become clear now that tRNA participates in several cellular functions other than protein synthesis such as priming tumour virus DNA synthesis, regulation of cognate amino acid biosynthesis operon and aromatic amino acid transport (Rich and Rajbhandary, 1976; Buck and Griffiths, 1981).

### Nature of modified bases in tRNA

The presence of modified bases in RNA was first detected in transfer RNA molecules, and methylation was one of the earliest detected modifications (Borek and Srinivasan, 1966; Borek, 1963). From then on, a large variety of modified bases have been detected in tRNA. These results have been summarised, in several review articles (Srinivasan and Borek, 1964; Nishimura, 1972; Nau, 1976; Feldman, 1977; Bjork, 1983).

Macromolecules offer a variety of complex structures which serve as substrates for methylation. Moreover the sites of methylation within each base is also varied. For instance, in uracil, C5-methylation is achieved in 5-methyl uracil, and this is the site for various other post-transcriptional modifications like 5-methoxycarbonyl methyl uridine (mcm<sup>5</sup>U) Uridine-5, oxyacetic acid methyl ester (mV) and 5-carboxy methyl amino-methyl uridine (cmnm<sup>5</sup>U). The C4-S bond is formed to get 4-thiouridine and N3 is involved in forming 3-(3-amino-3-carboxy propyl) uridine (acp<sup>3</sup>U).

In cytosine the C5-CH<sub>3</sub> is formed in 5-methylcytosine (m<sup>5</sup>C), N4 is involved in forming N<sup>4</sup>-acetyl cytidine (ac<sup>4</sup>C). N3 in 3-methyl cytidine (m<sup>5</sup>C) and C2 in forming 2-thiocytidine (s<sup>2</sup>C).

Adenine nitrogens as well as carbons can serve as sites for modification. For example N-1 and N-6 form 1-methyl adenine and 6-methyl adenine, N<sup>6</sup>-isopentenyl adenosine (i<sup>6</sup>A), 2-methyl thio-N<sup>6</sup>-isopentenyl adenosine (ms<sup>2</sup>i<sup>6</sup>A), (N-(9- $\beta$ -D-ribofuranosyl purin-6-yl) carbamoyl) threonine (t<sup>6</sup>A) respectively.

In the case of guanine, N-1, N-2, N-7 can be methylated to form 1-methyl guanine (m<sup>1</sup>G), 2-methyl guanine (m<sup>2</sup>G), N<sup>2</sup>N<sup>2</sup>-dimethyl guanine (m<sup>2</sup><sub>2</sub>G) and 7-methyl guanine (m<sup>7</sup>G). Hypermodification of guanine occurs in the case of base Y and its derivatives. The Q base or quinine is another derivative of guanine where N-7 is replaced by C-7 with a bulky side chain including a mannosyl or D-galactosyl group. Of these variety of modifications the most common is methylation of base and sugar moiety. The structure of all the various modified bases found in tRNA have been compiled by Nishimura (1979).

The nature and position of modified bases in tRNA are species-specific. Thus there are several bases which are exclusively found in eukaryotes or prokaryotes. Table 1 lists some of such bases. Thiolation, for instance is found only in prokaryotes whereas methylation of cytosine is restricted to eukaryotes. Besides, the abundance of certain modified bases at specific positions within the cloverleaf structure of tRNA varies distinctly between eukaryotes and prokaryotes. Figure 1 depicts these differences for each possible modification at a given position. Note the difference in case of

Table 1. Presence of minor nucleosides.

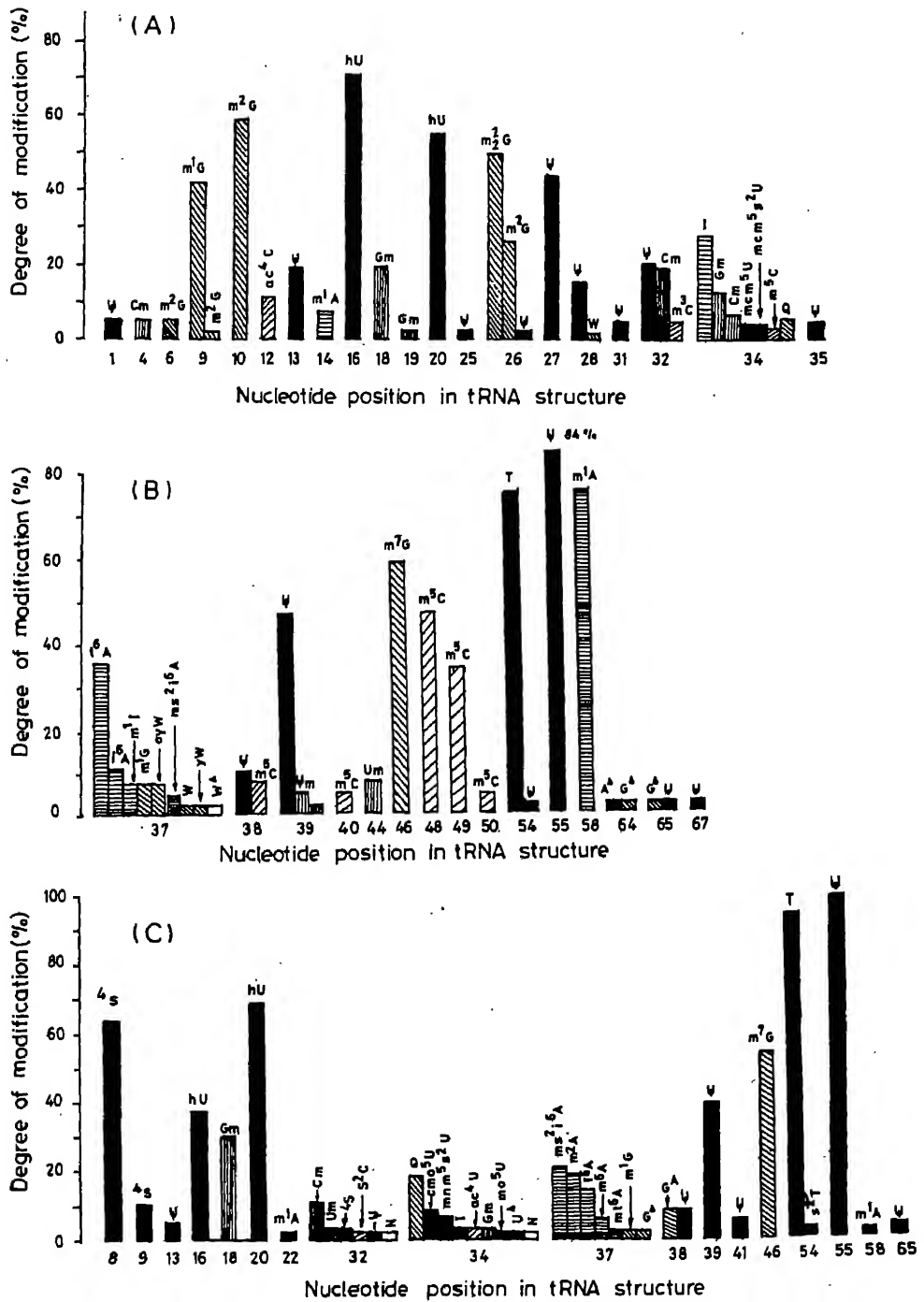
Exclusively in prokaryotic tRNAs	Exclusively in eukaryotic tRNAs
s <sup>2</sup> C	m <sup>3</sup> C
s <sup>4</sup> U	m <sup>4</sup> C
o <sup>5</sup> U	mcm <sup>5</sup> U
mo <sup>5</sup> U	Tm
cmo <sup>5</sup> U	m <sup>2</sup> G
mam <sup>5</sup> s <sup>2</sup> U	m <sup>2</sup> <sub>2</sub> G
cmam <sup>5</sup> U	i <sup>6</sup> A
cmam <sup>5</sup> s <sup>2</sup> U	m <sup>1</sup> I
mcmo <sup>5</sup> U	Wye and derivatives
s <sup>2</sup> T	
m <sup>2</sup> A	
m <sup>6</sup> A	
ms <sup>2</sup> i <sup>6</sup> A	

dihydrouridine at 16th position and 1-methyladenine at 58th position. It is clear that tRNAs from eukaryotes are modified to a greater extent than those from prokaryotes. Thus tRNA<sup>Ser</sup> from rat liver has 14 modified nucleotides whereas that from *Escherichia coli* has only seven modified nucleosides. However, the abundance of ribothymidine-54, m<sup>7</sup>G-46 and dihydrouridine-20 are comparable between the two groups (figure 1). Almost all bacterial tRNA contain m<sup>5</sup>U-54, exception to this are the tRNA from *Mycoplasma* (Walker and Rajbhandary, 1978), *Streptomyces* (Kuchino *et al.*, 1982), *Staphylococcus epidermis* (Roberts, 1974), archaeobacteria (Gupta and Woese, 1980), *Micrococcus luteus* (Delk *et al.*, 1976), *Thermus thermophilus* (Watanabe *et al.*, 1976a) and *Mycobacterium smegmatis* (Vani *et al.*, 1979). The transfer RNA from *Mycobacterium smegmatis* contains 1-methyladenine which is very abundant at 58th position of tRNA from eukaryotes but not in the tRNA from prokaryotes. The tRNA from *B. subtilis* (Raettig *et al.*, 1977), *B. stearothermophilus* and archaeobacteria (Gupta and Woese, 1980) are also reported to contain 1-methyl adenine. Archaeobacteria are also known to lack m<sup>7</sup>G in their tRNA (Woese and Fox, 1977).

#### Methods for detection and distribution of modified bases in tRNA

There are several methods used for the detection of modified bases in tRNA. The monomers derived from tRNA samples, after enzymatic or chemical digestion are analysed by two dimensional chromatography on cellulose coated glass plates (Nishimura *et al.*, 1967). There are several solvent systems given for developing the chromatogram. The one given by Feldman and Falter (1971) is specifically suited for acid labile residues, whereas, that given by Rogg *et al.* (1976) is suited for nucleoside separation.

A column chromatographic method has been given by Katz and Dudock (1969). A sensitive automatic recording system hooked to a column of cation resin was developed



**Figure 1.** Abundance of modified bases in tRNA. (A) and (B) eukaryotic tRNA (C) prokaryotic tRNA. Taken from Singhal and Fallis (1979).

y Uziel *et al.* (1968). Use of Dowex column in base separation was given by Leboy (1971).

Two dimensional finger printing (Sanger *et al.*, 1965) and paper electrophoresis technique (Brownlee and Cartwright, 1977) have also been used in the detection of modified bases.

More recently nuclear magnetic resonance (NMR) has been employed in the analysis of modified bases (Kastrup and Schmidt, 1978). High pressure liquid chromatography (HPLC) has become one of the most sensitive techniques for modified base analysis. Fractionation of modified nucleosides on HPLC using a column of  $\mu$ -Bondapak C18 has been reported by Davis *et al.* (1979) and Gehrke *et al.* (1978). With its high sensitivity HPLC can effectively be used for quantitation of modified residues in a given sample. The structure of modified bases has been worked out using mass spectrometry. Mass spectrometry has been used for detection of modified bases also (McCloskey, 1974; Von Mindin and McCloskey, 1973; Yamaizumi *et al.*, 1979).

Incorporation of radioactivity into tRNA molecules in the form, for instance, of [ $^{32}\text{P}$ ]-has contributed greatly to the sensitivity of detection. In cases where the incorporation of radioactivity in the form of precursors has been difficult, post-labelling techniques have been used. Randerath *et al.* (1972) have given the method for post-labelling the nucleosides with tritium and analysing by chromatography. Yet another post-labelling technique has been developed by Silberklang *et al.* (1977) wherein the RNA is digested into 3'-nucleoside triphosphates and they are 5'-labelled using  $\gamma$ -[ $^{32}\text{P}$ ]-ATP and the resulting labelled nucleoside diphosphates ( $^{32}\text{P}$  Np) are analysed. This apart, in the late 1970s several post-labelling techniques have been developed for sequencing tRNA (Lockard *et al.*, 1978; Gupta and Randerath, 1979; Kuchino *et al.*, 1979). These techniques involve the 3'-end labelling of tRNA, with  $^{32}\text{pCp}$  and T4 RNA ligase or 5'-end labelling using  $\gamma$ -[ $^{32}\text{P}$ ]-ATP and T4 polynucleotide kinase.

However the localization of modified bases within the cloverleaf structure of tRNA just by analysing various tRNAs in mixture, has not been possible. The exact position of modified bases is known only while sequencing pure single species of tRNA. By adopting tRNA sequencing method of Kuchino *et al.* (1979), for post labelling and analysing tRNA mixtures, we have been able to empirically localize the modified bases within the cloverleaf structure (Nishimura, S., personal communication; Vani, 1982). The oligonucleotides derived after digestion of tRNA mixtures with formamide are labelled at 5'-ends with  $\gamma$ -[ $^{32}\text{P}$ ]-ATP and fractionated on sequencing gels. The oligonucleotide bands can now be assigned empirically to various regions of the cloverleaf structure of tRNA. The data from one such analysis are shown in figure 2 and table 2.

The data on the nucleotide sequence of a large number of tRNA species indicates the probable positions at which modified bases can occur. A compilation of such data correlated with the nature of hydrogen bonding each of them can involve in, brings out certain interesting features of modified base distribution. From the data given in table 3, certain features of the distribution of modified bases in tRNA can be elucidated: (i) Pseudouridine and 5-methylcytosine, show the maximum variation in their position of occurrence, and both of these bases can involve in Watson-Crick base pairing. (ii) Certain bases like 1-methyl guanine, 1-methyl inosine, 2-thio-cytosine, 2-dimethyl

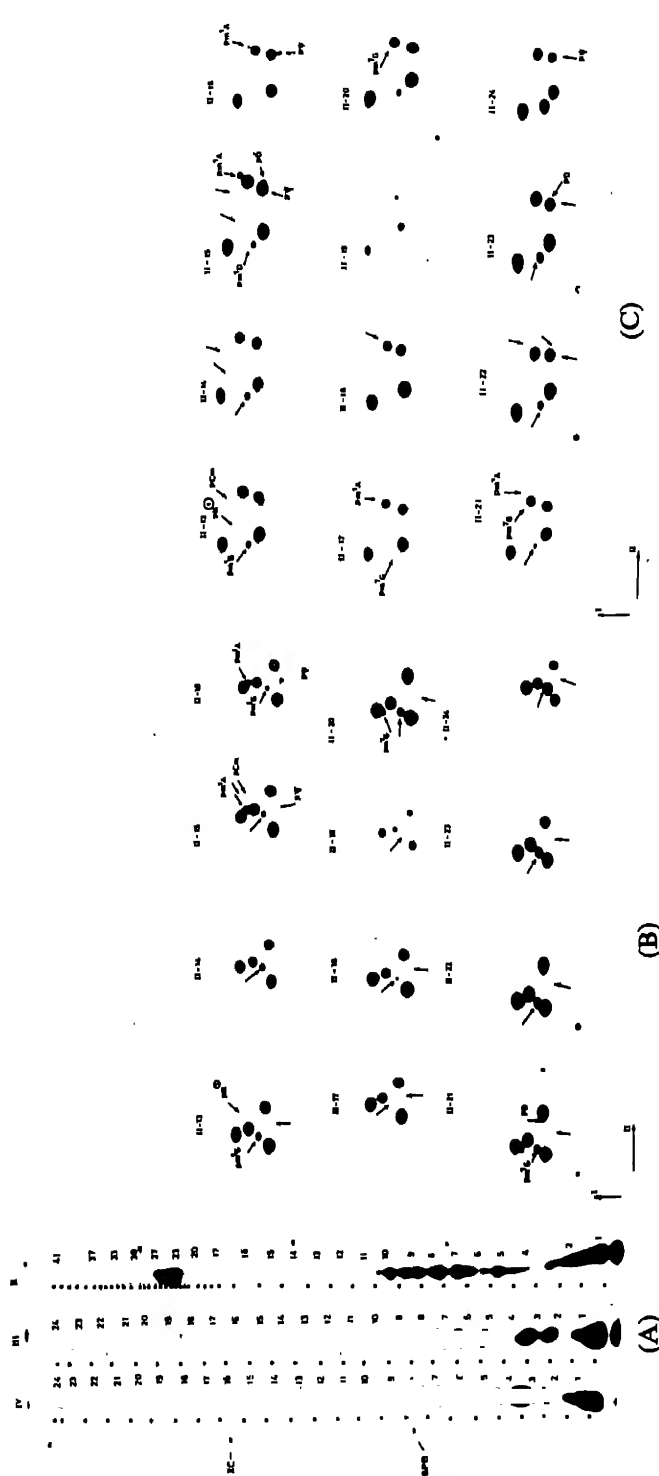


Figure 2. Localization of modified bases within tRNA structure by post labelling. (A) Analysis of oligonucleotides derived from tRNA mixtures on sequencing gels after labelling at 5'-end with  $\gamma$ - $[^{32}\text{P}]$ -ATP. (B) and (C) Analysis of mononucleotides derived from fraction II on the gel (A). The number II-13 to II-24 correspond to the band numbers indicated on (A), lane II. (B) In solvent system A; I, Isobutyric acid: 0.5 M ammonium hydroxide 5:3 v/v; II, Isopropanol: concentrated HCl: Water 70:15:15 v/v/v. (C) In solvent system B; I, Isobutyric acid, ammonia, water 60:1:33 v/v/v; II, 0.1 M sodium acetate pH 6.8, ammonium sulphate and *n*-propanol 100:60 gms: 2 v/v/v.

**Table 2.** Localisation of the oligonucleotides to the different regions of tRNA structure and analysis modified base composition of fraction II.

Band number	Corresponding region of tRNA	Modified base(s) present
1-8	Stem (a)	pm <sup>1</sup> G and pCm
9-11	Stem (e)	pm <sup>1</sup> G
12-16	Loop (iv) and Stem (e)	pψ, pCm, pm <sup>1</sup> G, pN <sup>1</sup> , pm <sup>1</sup> A and pD.
17-21	Stem (d), Loop III and Stem (c)	pm <sup>1</sup> G, pm <sup>7</sup> G, pm <sup>1</sup> A.
22-27	Loop (II)	pm <sup>1</sup> G, pψ and pN <sup>3</sup>
28-31	Stem (c)	pm <sup>1</sup> G
31-39	Stem (b) and Loop I	pm <sup>1</sup> G, pN <sup>2</sup>
39-44	Stem (a)	None

Nomenclature for the regions of the cloverleaf is as given by Clark (1977). pm<sup>1</sup>G-1-methylguanosine 5'-monophosphate, pCm-2'-O-methylcytosine 5'-monophosphate, ψ *p*-pseudouridine 5'-monophosphate, pm<sup>1</sup>A-1-methyladenine 5'-monophosphate, pD-dihydrouridine 5'-monophosphate, pm<sup>7</sup>G-7-methylguanosine 5'-monophosphate. pN<sup>1</sup>, pN<sup>2</sup>, pN<sup>3</sup>-unidentified nucleotides.

guanine, 1-methyl adenine can involve only in Hoogsteen base pairing but not Watson-Crick base pairing. Concurrently they occur only in either the loop or the junction regions which does not demand conventional Watson-Crick pairing. However m<sup>2</sup>G in tRNA of yeast, *Bombyx mori* and mammalian tRNA occurs at the 10th position within the D-stem (Kuntzel *et al.*, 1974; Sprague *et al.*, 1977; Chen and Roe, 1978). (iii) Hyper-modified bases like t<sup>6</sup>A, i<sup>6</sup>A, Y, O<sub>2</sub>YW and alkylated cytosine like m<sup>3</sup>C occur exclusively in the looped out regions. These bases cannot be involved in either Watson-Crick or Hoogsteen type of hydrogen bonding. Thus a correlation between the region of occurrence of modified bases and their ability to form hydrogen bonding is reflected in the distribution of modified bases along the sequence of transfer RNA. However it should be noted that all modified bases can involve in stacking interactions if not hydrogen bonding, even with sequentially distant residues, thus maintaining the tertiary structure of tRNA.

### Enzymes of tRNA modification/methylation

As early as 1963 Mandel and Borek demonstrated that the methyl groups of all the methylated nucleotides come from methionine, including the -CH<sub>3</sub> group of ribothymidine. The enzymes which mediate this reaction *in vivo* constitute the group of S-adenosyl methionine-tRNA methyltransferases (EC 2.1.1. 29-36) generally referred to as tRNA methylases.

The variety seen in tRNA modification demands the presence within the cell of a whole battery of enzymes with unique base and site specificity. The presence of six different methylases in *E. coli* was shown by Hurwitz *et al.* (1964). They identified uracil,

Table 3. Nature of the possible base pairing of modified bases present in transfer RNA.

Nature of Modified nucleoside	Position(s) of occurrence in tRNA	Nature of base pair	
		Watson-Crick	Hoogsteen
s <sup>4</sup> U	8 <sub>i</sub>	+	+
o <sup>5</sup> U	34 <sub>i</sub>	+	+
ψ	27 <sub>s</sub> /28 <sub>s</sub> /32 <sub>i</sub> /38 <sub>i</sub> /39 <sub>s</sub> /40 <sub>s</sub> /55 <sub>i</sub> /65 <sub>s</sub>	+	+
U <sub>m</sub>	32 <sub>i</sub>	+	+
D	17 <sub>i</sub>	+	+
s <sup>2</sup> T	54 <sub>i</sub>	+	+
mo <sup>5</sup> U	34 <sub>i</sub>	+	+
T	54 <sub>i</sub>	+	+
s <sup>2</sup> C	32 <sub>i</sub>	-	+
m <sup>2</sup> C	34 <sub>i</sub> /38 <sub>i</sub> /40 <sub>s</sub> /48 <sub>v</sub> /49 <sub>s</sub> /50 <sub>s</sub>	+	+
ac <sup>4</sup> C	34 <sub>i</sub> , 12 <sub>s</sub>	+	+
m <sup>3</sup> C	32 <sub>i</sub>	-	-
m <sup>2</sup> A	37 <sub>i</sub>	+	+
m <sup>1</sup> A	9 <sub>i</sub> /14 <sub>i</sub> /58 <sub>i</sub>	-	+
m <sup>6</sup> A	14 <sub>i</sub> /37 <sub>i</sub>	-	-
i <sup>6</sup> A	37 <sub>i</sub>	-	-
m <sup>1</sup> G	9 <sub>i</sub> , 37 <sub>i</sub>	-	+
m <sub>2</sub> <sup>2</sup> G	26 <sub>j</sub>	-	+
m <sup>2</sup> G	10 <sub>s</sub>	-	+
G <sub>m</sub>	39 <sub>s</sub>	+	+
m <sub>2</sub> <sup>2</sup> G	26 <sub>j</sub>	-	+
m <sup>7</sup> G	46 <sub>v</sub>	+	-
Q	34 <sub>i</sub>	+	-
yW	37 <sub>i</sub>	-	-
O <sub>2</sub> yW	37 <sub>i</sub>	-	-
m <sup>1</sup> I	37 <sub>i</sub>	-	+

s—stem, l—loop, j—junction, v—variable loop, (+)—possible, (—)—not possible.

cytosine, adenine tRNA methylases and three enzyme fractions which methylated guanine—one being specific for 7-position and two for N-1 of guanine.

The purification of modifying enzymes has been difficult since they are present in low amounts. Further, the lack of suitable tRNA substrates for each enzyme and the instability of the enzymes have hampered the extensive characterization of the enzymes. Most enzymes so far characterised are known to be composed of one polypeptide in the active state, except tRNA (m<sup>5</sup>U) methyltransferase from *S. faecalis* which is composed of two identical subunits (Bjork, 1983). For this methylation reaction methyl tetrahydrofolate is used as methyl group donor in *S. faecalis* as well as *B. subtilis*. The tRNA (s<sup>4</sup>U) synthetase is also known to be composed of at least two subunits (Abrell *et al.*, 1971).

Methylases have been partially purified from several systems. Aschoff *et al.* (1976) have carried out the purification of 7-methyl guanine methylase from *E. coli*. They have used tRNA<sup>fmet</sup> of *B. subtilis* which lacks m<sup>7</sup>G, as the substrate. From *E. coli* strains, tRNA m<sup>1</sup>G-methyl transferase has been purified by Hjalmarsson *et al.* (1983) and quenosine

inserting enzyme by Okada and Nishimura (1979). The supK methylase has been studied by Pope and Reeves (1978). The association of methylase with virions was first reported by Gantt *et al.* (1971) in avian myeloblastosis virus (AMV). Purification of this enzyme was done by Taylor and Gantt (1979) to about 1000 fold. They have shown that the enzyme methylates G-10 of *E. coli* B tRNA<sup>Phe</sup> and has a molecular weight of 77,000.

In the purification of methylases, apart from ion exchange and gel filtration techniques, blue sepharose column chromatography has been employed by Greenberg and Dudock (1980) and it has been possible to carry out the purification of tRNA-m<sup>1</sup>A methyltransferase I from *Mycobacterium smegmatis* by affinity chromatography on AMP-sepharose column (Vani and Ramakrishnan, 1984a). Studies on substrate specificity and partial purification of the 1-methyl adenine methyl transferase from rat liver were carried out by Kuchino and Nishimura (1974). The recognition sequence of the enzyme has been suggested to be G-T-ψ-C-G-A-A-U-C in the TψC loop region. Glick and Leboy (1977) have also investigated the nature of 1-methyl adenine methylase of rat liver and shown that it methylates A-58. 1-Methyl adenine methylase and 2-methyl guanine methylase from HeLa cells were partially purified and shown to methylate A-58 and G-27 of *E. coli* (Spermulli *et al.*, 1974). At least one enzyme for each modification has been detected in all the systems studied to date. However there are reports indicating the presence of more than one enzyme for the same modification but with different target specificity. Smolar *et al.* (1975) have shown that in yeast there exist two tRNA (m<sup>1</sup>G) methyltransferases; one enzyme catalyzing the formation of m<sup>1</sup>G at position 37 and the other at position 9. There is also a similar report on tRNA (m<sup>2</sup>G) methyltransferase from rat liver (Kraus and Staehelin, 1974). They showed that one of them recognises a sequence like Y-G-Cp, the other Y-G-Up. It is interesting to note that these two enzymes have been reported to have different sensitivity to S-adenosyl homocysteine (Glick *et al.*, 1975).

The occurrence of more than one enzyme species has also been reported for tRNA (m<sup>1</sup>A) methyltransferase from rat brain cortex (Salas and Dirheimer, 1979) and *Mycobacterium smegmatis* (Vani and Ramakrishnan, 1984a). In *M. smegmatis*, two activities of tRNA (m<sup>1</sup>A) methyl transferase have been demonstrated, one methylating both *E. coli* and yeast tRNA, and the other methylating only the *E. coli* tRNA. These two activities have different sensitivity to methylation inhibitors like S-adenosyl homocysteine and an analog SIBA (5'-S-isobutyl-thioadenosine).

The influence of polyamines and salts on methylases have been investigated in several cases. Leboy (1971) showed that spermine, spermidine, putrescine and salts like ammonium acetate and magnesium acetate have differential effects on different base specific methylations. Leboy and Glick (1976) showed that tRNA m<sup>2</sup>-G-methyltransferase I had optimum concentrations of spermidine about ten times and of putrescine about twice as much as was required for tRNA-m<sup>2</sup>G methyltransferase II activity. Monovalent cations like potassium, sodium and ammonium are also known to have an effect on tRNA methylase activity (Agris *et al.*, 1975).

All known tRNA modifications, except the formation of Q-base, occur at polynucleotide level. The Q-base is inserted into tRNA by tRNA transglycosylase, which replaces guanine with quinine (Okada and Nishimura, 1979). In most RNA transmethylation, reactions S-adenosyl methionine (AdoMet) is the methyl donor. However in certain grampositive bacteria like *B. subtilis* and *Streptococcus faecalis* tRNA (m<sup>5</sup>U)



methyltransferase utilizes, 5,10-methylene tetrahydrofolate as the methyl donor (Kersten *et al.*, 1975; Delk and Rabinowitz, 1975). However, it is interesting to note that *B. subtilis* tRNA lacking m<sup>5</sup>U accepts methyl groups from tRNA (m<sup>5</sup>C) methyltransferase from *E. coli* using AdoMet as the methyl donor (Arnold *et al.*, 1976). Similarly, *E. coli* tRNA deficient in m<sup>5</sup>U can act as substrate for the enzyme from *B. subtilis* (Kersten *et al.*, 1975). These observations indicate that although the enzyme from these two sources need different cofactors, they have very similar specificity and mechanism of recognition. In fact the studies of Gambaryan *et al.* (1979) have shown that the initial binding of tRNA methyltransferases is rather non-specific, meaning that they recognise a general structural feature of tRNA and subsequently methylate a given base at a defined position in a highly specific manner. These observations were made by using immobilized tRNA.

The maturation of tRNA molecules is a highly ordered process. The size reduction and modification are intimately related processes during the maturation of tRNA precursors. The level of certain tRNA modifying enzymes within the cell is regulated by factors like growth rate. For instance the level of tRNA (m<sup>5</sup>U) methyltransferase increases with increasing growth rate, while that of tRNA (m<sup>1</sup>G) methyltransferase and tRNA (mnm<sup>5</sup>s<sup>2</sup>U) forming enzyme remain almost constant (Ny and Bjork, 1977, 1980; Ny *et al.*, 1980). Temperature is known to influence the extent of modification of tRNA in certain species like *Bacillus thermophilus* and *Thermus thermophilus* (Agris *et al.*, 1973; Watanabe *et al.*, 1976b).

### Functions of modified bases in tRNA

The search for a function for the specific alkylation of tRNA bases has been on since the discovery of modified bases in tRNA. But this particular aspect has somehow been elusive to any unique generalisation.

One of the correlations drawn is between the nature of the residue at the position 37 in the tRNA structure and the first base of the codon (Nishimura, 1972, 1979). For instance, hydrophobic nucleosides such as i<sup>6</sup>A or its derivatives are almost always found in tRNAs recognising codons starting with uracil and the tRNA interacting with codons starting with adenine have hydrophilic residues at this position. However, similar correlation is not found with codons starting with guanine or cytosine. Thus it has been suggested by Nishimura (1972, 1979) that probably the hypermodification is necessary to strengthen the fidelity of translation. It has been observed (Gefter and Russell, 1969) that tRNA<sup>Tyr</sup> from *E. coli* deficient in ms<sup>2</sup>i<sup>6</sup>A binds less efficiently to the ribosomes. In yeast the antisuppressor mutations sin 1 and mod 5-1 reduce the activity of serine-inserting UGA suppressor and the tyrosine-inserting UAA suppressor respectively and it was found that both these strains completely lack the i<sup>6</sup>A normally present in position 37 of the suppressor tRNAs (Laten *et al.*, 1978; Janner *et al.*, 1980). Both mutants however grow relatively well, showing only 10% reduction in growth rate. *E. coli* tRNA<sup>Phe</sup> carrying i<sup>6</sup>A37 in place of ms<sup>2</sup>i<sup>6</sup>A37 is known to be less active in ribosome binding (Hoburg *et al.*, 1979). Yeast tRNA<sup>Phe</sup> lacking its usual component yW at 37 fails to bind to ribosomes and carry out poly(U) directed *in vitro* translation (Thiede and Zachau, 1968). Conformational studies with i<sup>6</sup>A and t<sup>6</sup>A monomers have

indicated that the side chains might block the two *N*-sites of adenine for hydrogen bonding resulting in the inability to form Watson-Crick base pairing (Bugg and Thewalt, 1972; Parthasarathy *et al.*, 1974). These observations support the hypothesis that these kinds of modifications promote and maintain single stranded structure in the anticodon loop.

Mutation *his T*, leading to the absence of pseudouridine at the position 38–39 is known to lead to the loss of regulation of histidine operon (Roth *et al.*, 1966). Further it has been revealed that tRNA<sup>His</sup> lacking  $\psi$  reads the seven histidine codons present in a row in the histidine leader mRNA inefficiently (Johnston *et al.*, 1980). Yet another link between amino acid biosynthesis and tRNA base modification was shown recently by Bjork (1980). All mutants defective in the common pathway of aromatic amino acids are deficient in  $\text{cmo}^5\text{U}$  and  $\text{mcmO}^5\text{U}$  in their tRNAs. It has also been demonstrated that the deficiency of thiomethyl group of  $\text{ms}^2\text{i}^6\text{A}$  in tRNA stimulates the transport of aromatic amino acids (Buck and Griffiths, 1981).

In yeast *trm-1* mutation leading to the deficiency of  $\text{m}_2^2\text{G}$  at position 26 in tRNA leads to a slight reduction in growth rate. Further the tRNA<sup>Ser</sup> from mutants has reduced efficiency in *in vitro* charging (Bjork and Kjellin-Straby, 1977). Specific chemical reduction of  $\text{m}^7\text{G}$  46 disrupts the C13-C22- $\text{m}^7\text{G}$ 46 interaction leading to a slightly less ordered tRNA structure (Arcari and Hecht, 1978). *E. coli* mutant *trm B* defective in  $\text{m}^7\text{G}$  formation is indicated to show slower growth than *trm B*<sup>+</sup> and heterologous *in vitro* methylation producing  $\text{m}^7\text{G}$  or  $\text{m}^2\text{G}$  results in an altered kinetics of aminoacylation (Hoburg *et al.*, 1979; Roe *et al.*, 1973). The effect of modification on aminoacylation of tRNA has not been obvious in all systems studied. However in rats the undermethylated tRNA, extracted after feeding a diet containing ethionine, shows reduced levels of aminoacylation; when injected into *Xenopus* oocytes, these tRNAs are found to have reduced charging capacity (Ginzburg *et al.*, 1979). Studies with *E. coli* and yeast undermethylated tRNA species have led to the conclusion that methylated tRNA is better substrate in a wider range of heterologous systems (Peterkofsky, 1964). Similar studies with reference to the presence of 1-methyladenine have also shown that the presence of this modification render the tRNA a better substrate for aminoacylation in heterologous system (Vani and Ramakrishnan, 1984b). The presence of  $\text{m}^5\text{U}$  seems to influence the elongation factor directed *A*-site binding (Kersten *et al.*, 1981). The effect of ribothymidine present in mammalian tRNA on *in vitro* protein synthesis in the presence of certain of the elongation factors has been shown by Roe and Tsen (1977). Kersten *et al.* (1981) have shown that when ribothymidine is absent in tRNA there is misincorporation of leucine during poly(U) directed protein synthesis *in vitro*, the effect is pronounced on misincorporation by tRNA<sub>4</sub><sup>Leu</sup> and does not induce any other tRNA<sup>Leu</sup> to misread. The stability of tRNA<sup>Met</sup> is higher, when  $\text{s}^2\text{m}^5\text{U}$ 54 is present as compared to that in the presence of  $\text{m}^5\text{U}$ 54 which is further lowered when only U54 is present (Davenloo *et al.*, 1979).

Transfer RNA modification might serve as a fine control mechanism for modulating translational efficiency. During the development of slime mold *Dictyostelium discoideum* and the amphibian *Rana catibeinna*, changes occur in the tRNA modification pattern (Palatnik *et al.*, 1977; Klee *et al.*, 1978; Dingermann *et al.*, 1977). In *D. discoideum* the levels of  $\text{m}^5\text{U}$ , and  $\text{m}^5\text{C}$  decrease during development from vegetative cells to spore formation (Dingermann *et al.*, 1977) and  $\text{m}^5\text{U}$  containing tRNA species

are preferentially used in protein synthesis (Dingermann *et al.*, 1980). Further, changes in the formation of *Q*-base also occurs during development of *D. discoideum* (Palatnik *et al.*, 1977). Transfer RNA from tumour tissue has exclusively guanine instead of *Q*-base in the Wobble position of tRNA<sup>Tyr</sup>, tRNA<sup>His</sup>, tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup>. Recently studies with tobacco mosaic virus have shown that tRNA<sup>Tyr</sup> containing *Q*-base prevents read-through whereas that with guanine in place of quinine leads to the synthesis of a read-through product, a 160 K protein instead of 110 K protein (Bienz and Kubli, 1981).

During *Drosophila* development the content of *Q*-base changes dramatically but this is influenced by factors such as growth medium and temperature of cultivation (White *et al.*, 1973; Worsnick and White, 1977). Thus the pattern of synthesis of certain modified bases changes during developmental processes in eukaryotes.

The application of modern techniques of molecular biology coupled with the studies using well-defined mutants defective in tRNA modification will certainly lead to better understanding of the formation and function of modified nucleosides in RNA. The process is complex and is interlinked with several aspects of cellular metabolism at various levels. Thus the presence of modified nucleosides seems to be necessary for the subtle tuning and coordination of tRNA function as well as specific interactions with several protein factors.

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## Sodium requirement and metabolism in nitrogen-fixing cyanobacteria

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**Abstract.** Sodium affects the metabolism of eukaryotes and prokaryotes in several ways. This review collates information on the effects of  $\text{Na}^+$  on the metabolism of cyanobacteria with emphasis on the  $\text{N}_2$ -fixing filamentous species.  $\text{Na}^+$  is required for nitrogenase activity in *Anabaena torulosa*, *Anabaena* L-31 and *Plectonema boryanum*. The features of this requirement have been mainly studied in *Anabaena torulosa*. The need for  $\text{Na}^+$  is specific and cannot be replaced by  $\text{K}^+$ ,  $\text{Li}^+$ ,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . Processes crucial for expression of nitrogenase such as molybdenum uptake, protection of the enzyme from oxygen inactivation and conformational activation of the enzyme are not affected by  $\text{Na}^+$ . Mo-Fe protein and Fe protein, the two components of nitrogenase are synthesized in the absence of  $\text{Na}^+$  but the enzyme complex is catalytically inactive. Photoevolution of  $\text{O}_2$  and  $\text{CO}_2$  fixation, which are severely inhibited in the absence of  $\text{Na}^+$ , are quickly restored by  $\text{NH}_4^+$ , glutamine or glutamate indicating that  $\text{Na}^+$  deprivation affects photosynthesis indirectly due to deficiency in the products of  $\text{N}_2$  fixation.  $\text{Na}^+$  deprivation decreases phosphate uptake, nucleoside phosphate pool and nitrogenase activity. These effects are reversed by the addition of  $\text{Na}^+$  suggesting that a limitation of available ATP caused by reduced phosphate uptake results in loss of nitrogenase activity during  $\text{Na}^+$  starvation.

$\text{Na}^+$  influx in *Anabaena torulosa* and *Anabaena* L-31 is unaffected by low  $\text{K}^+$  concentration, is carrier mediated, follows Michaelis-Menten kinetics and is modulated mainly by membrane potential. Treatments which cause membrane depolarisation and hyperpolarisation inhibit and enhance  $\text{Na}^+$  influx respectively. These cyanobacteria exhibit rapid active efflux of  $\text{Na}^+$ , in a manner different from the  $\text{Na}^+/\text{H}^+$  antiporter mechanism found in *Anacystis nidulans*.

$\text{Na}^+$  requirement in nitrogen metabolism including nitrate assimilation, synthesis of amino acids and proteins, in respiration and oxidative phosphorylation, in transport of sugars and amino acids, cellular distribution of absorbed sodium, physiological basis of salt tolerance and prospects of reclamation of saline soils by cyanobacteria are the other aspects discussed in this review.

**Keywords:** Cyanobacteria: sodium requirement; nitrogen fixation; sodium transport; salt tolerance.

### Introduction

Sodium influences the metabolism of microbes, plants and animals. The food we eat is tasteless without sodium chloride, but apart from this gastronomical role,  $\text{Na}^+$  is a key element in regulating a variety of metabolic processes in animal cells including

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Abbreviations used: CCCP, Carbomylcyanide m-chlorophenyl-hydrazone; DCCD, N,N'-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP, dinitrophenol.

responses which underlie the action potentials of nerve axons (Hodgkin, 1964). There is abundant evidence from mammalian systems that the sodium pump generated by  $\text{Na}^+$ ,  $\text{K}^+$  ATPase, in addition to expelling excess  $\text{Na}^+$ , effects a circulation of  $\text{Na}^+$  and supports transport of inorganic and organic metabolites (Heinz, 1974; Harold, 1982; Kimmich, 1982). Moreover, transmembrane  $\text{Na}^+$  fluxes have been shown to be involved in ATP synthesis (Hallam and Whittam, 1977). ATPases stimulated by  $\text{Na}^+$  and  $\text{K}^+$  have also been demonstrated in plant plasmalemma preparations but their precise functions are not well understood (Harold, 1977). Recent work suggests that electrogenic proton translocating ATPases isolated from plant microsomal membranes are involved in the active transport of various solutes (Poole, 1978; Sze and Churchill, 1983). In addition to proton extruding ATPase(s) vectorial redox reactions facilitate such primary solute transport in bacteria (Harold, 1982; Elferink *et al.*, 1984).

Distinct from the enzyme-linked primary transport involving  $\text{Na}^+$  and related processes, secondary active transport such as the  $\text{Na}^+/\text{H}^+$  antiporter, first predicted by Mitchell (1966), has been since elucidated (Krulwich, 1983; Heinz and Grassel, 1984). The  $\text{Na}^+/\text{H}^+$  antiporter which catalyses translocation of  $\text{Na}^+$  and  $\text{H}^+$  in opposite directions may operate in either direction across the cell membrane. Moreover, movement of  $\text{Na}^+$  ions coupled to another metabolite in the same direction by the symport mechanism also facilitates substrate transport. The  $\text{Na}^+/\text{H}^+$  exchange reactions have been found widely in prokaryotic and eukaryotic membranes. At least some prokaryotes possess a membrane potential-dependent  $\text{Na}^+/\text{H}^+$  antiporter which catalyses  $\text{Na}^+$  extrusion in exchange for  $\text{H}^+$  whereas in other bacteria the antiporter may function electroneutrally at appropriate external pH values. The bacterial  $\text{Na}^+/\text{H}^+$  antiporter thus constitutes a critical component of  $\text{Na}^+$  circulation designed to maintain a diffusion potential of  $\text{Na}^+$  for use by  $\text{Na}^+$ -coupled bioenergetic processes (Krulwich, 1983). The prokaryotic antiporter is also involved in pH homeostasis in the alkaline pH range. In eukaryotes, an electroneutral  $\text{Na}^+/\text{H}^+$  antiporter has been found in a wide variety of cell and tissue types. The normal direction of this antiporter appears to be that of  $\text{Na}^+$  uptake and  $\text{H}^+$  extrusion which is implicated as a component of a complex  $\text{Na}^+$  circulation process. The antiporter also influences, in many experimental systems, internal pH which in turn could affect early events in a variety of differentiating and proliferating systems (Krulwich, 1983).

$\text{Na}^+$  is also involved in other metabolic processes. Many heterotrophic bacteria possess  $\text{Na}^+$ -sensitive decarboxylases. For instance, the oxaloacetate decarboxylase permits growth of *Salmonella typhimurium* and *Aerobacter aerogenes* on citrate only in the presence of  $\text{Na}^+$  (O'Brien and Stern, 1969a, b) and in *Klebsiella aerogenes* this decarboxylase functions as a  $\text{Na}^+$  translocase (Dimroth, 1980). The methylmalonyl-CoA decarboxylase involved in succinate fermentation in *Veillonella alcalescens* is specifically activated by  $\text{Na}^+$  (Hilpert and Dimroth, 1984).

A crucial role of  $\text{Na}^+$  is its contribution in causing salinity. Over 950 million hectares of land area of the earth are salt affected and nearly one-third of the irrigated land or 76.7 million hectares are estimated to be affected by salinity posing a serious threat to ecological balance and to the agricultural economy of mankind (Epstein, 1980). The ways in which plants cope with salt stress are not fully understood, although some aspects of salt tolerance are being exemplified. During salt stress plants as well as algae and bacteria resort to osmotic adjustment by building up high internal concentrations

of inorganic and/or organic solutes (Flowers *et al.*, 1977; Szalay and MacDonald, 1980). In plants, the inorganic ions are sequestered in vacuoles which occupy about 90 % of the mature cell volume. Another mechanism of sodium tolerance, as in animal cells, is the highly selective transport of potassium in plants (Epstein, 1980), algae and bacteria (Szalay and MacDonald, 1980).

From the foregoing it is clear that the influence of sodium on the life processes of various groups of organisms has received considerable emphasis. A striking exception to such major attention is the group of cyanobacteria or blue-green algae, prokaryotes capable of oxygenic photosynthesis which are widely prevalent in conventionally favourable climates of tropical and temperate regions as well as in extremes of heat and cold (Fogg *et al.*, 1973). They are abundant in brackish soils of coastal areas (Carter, 1933) and the  $\text{Na}^+$ -rich saline alkali soils (Singh, 1950). Such cyanobacteria have been used in reclamation of the latter type of soils of Northern India with some success (Singh 1961). Many cyanobacteria are agronomically important in tropical rice paddies where they fix atmospheric nitrogen and carbon using solar energy. A major recent development is the unequivocal demonstration that  $\text{Na}^+$  is essential for cyanobacterial nitrogenase activity (Apte and Thomas 1980, 1984).

In this article we shall collate the available information on the requirement and metabolism of sodium in cyanobacteria and stress the recent advances in sodium transport and nutrition especially its role in cyanobacterial nitrogen fixation. A great deal of the recent work described here relates to experiments carried out in our laboratory during the last five years. It compliments and extends earlier and current work in other laboratories.

## Sodium requirement for nitrogen assimilation in cyanobacteria

### General features

The entire taxonomic group of cyanobacteria, irrespective of their morphological organisation, ecological distribution and nature of carbon and nitrogen nutrition, requires  $\text{Na}^+$  for growth. During the last four decades there have been several reports indicating the need for  $\text{Na}^+$  especially in nitrate-grown cultures of many cyanobacteria such as the unicellular diazotrophs (incapable of  $\text{N}_2$ -supported growth) *Chroococcus* sp. (Emerson and Lewis, 1942), *Microcystis aeruginosa* (McLachlan and Gorham, 1961) and *Anacystis nidulans* (Kratz and Myers, 1955) as well as the diazotrophic (capable of  $\text{N}_2$ -supported growth) filamentous cyanobacteria *Anabaena variabilis*, *Nostoc muscorum* (Kratz and Myers, 1955), *A. flos-aquae* (Bostwick *et al.*, 1968) and *A. cylindrica* (Brownell and Nicholas, 1967). Whether  $\text{Na}^+$  is needed in the presence of other forms of combined nitrogen has not been examined sufficiently but we have recently found that growth of  $\text{NH}_4^+$ -supplemented cultures of two *Anabaena* spp. is not affected by  $\text{Na}^+$  starvation (S. K. Apte and Joseph Thomas, unpublished results).

Very little is known about the requirement of  $\text{Na}^+$  for  $\text{N}_2$ -fixing cultures of cyanobacteria except for certain *Anabaena* spp.. Thus Allen and Arnon (1955) and Brownell and Nicholas (1967) found that *A. cylindrica* specifically required  $\text{Na}^+$  during growth in the absence of combined nitrogen, while Apte and Thomas (1980, 1984) reported an absolute growth requirement of  $\text{Na}^+$  for  $\text{N}_2$ -fixing cultures of *A. torulosa*



(a brackish water species) and *Anabaena* L-31 (a fresh water species). The  $\text{Na}^+$  requirement is highly specific and the cation cannot be replaced by  $\text{K}^+$  (Allen and Arnon, 1955; Kratz and Myers, 1955; Bostwick *et al.*, 1968; Apte and Thomas, 1984)  $\text{Rb}^+$ ,  $\text{Cs}^+$  (Allen and Arnon, 1955)  $\text{Li}^+$ ,  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  (Apte and Thomas, 1984). The last authors also found that all the salts of  $\text{Na}^+$  tested supported growth irrespective of the accompanying anion.

The reported concentrations of  $\text{Na}^+$  required by cyanobacteria are quite low.  $\text{Na}^+$  requirement saturated at 1 ppm ( $\approx 17 \mu\text{M}$ )  $\text{NaCl}$  in fresh water cyanobacteria and at 100 ppm (1.7 mM) in marine forms (Batterton and Van Baalen, 1971). Allen and Arnon (1955) reported  $\text{Na}^+$  requirement in the range of 1–5 ppm (17–85  $\mu\text{M}$ ) while Kratz and Myers (1955) found a much higher requirement (680  $\mu\text{M}$ ). Recently Apte and Thomas (1984) demonstrated that about 20–25  $\mu\text{M}$  was the minimum level of  $\text{Na}^+$  required for detectable  $\text{N}_2$ -supported growth of the fresh water cyanobacterium *Anabaena* L-31 and the brackish water species *A. torulosa*. The concentrations required for optimum growth of the fresh water and the saline species have been found to be 0.5 mM and 20 mM respectively (S. K. Apte and Joseph Thomas, unpublished results). These concentrations are at least 10–20 times higher than those required for some of the known essential micronutrients of cyanobacteria (Wolk, 1973). The amount of  $\text{Na}^+$  needed has sometimes been found to vary with the nature of nitrogen nutrition. Thus, Brownell and Nicholas (1967) observed a much higher requirement (400  $\mu\text{M}$ ) for *A. cylindrica* during growth on nitrate as compared to only 4  $\mu\text{M}$  during growth on  $\text{N}_2$ . On the other hand we have noticed a much lower requirement ( $< 15 \mu\text{M}$ ) for *A. torulosa* during growth on nitrate compared to the amount (20 mM) required for optimum diazotrophic growth (S. K. Apte and Joseph Thomas, unpublished results).

In contrast to the requirement for low quantities of  $\text{Na}^+$  reported above, there are cases of halophilic cyanobacteria which need considerably high concentrations of  $\text{NaCl}$  (see section on salt tolerance). However, this requirement is for osmoregulation and not nutritional.

### Sodium requirement for nitrogen fixation

In diazotrophically grown *A. cylindrica*  $\text{Na}^+$  deficiency was found to decrease the incorporation of  $^{15}\text{N}_2$  but increase the incorporation of  $^{15}\text{NH}_4$  or [ $^{14}\text{C}$ ]-glutamate into protein (Brownell and Nicholas, 1967). Apte and Thomas (1980) clearly demonstrated the dependence of cyanobacterial nitrogenase activity on the presence of  $\text{Na}^+$ . In two heterocystous diazotrophs, *A. torulosa* and *Anabaena* L-31, it was shown that  $\text{Na}^+$  deficiency resulted in significant loss of nitrogenase activity. Readdition of  $\text{Na}^+$  to  $\text{Na}^+$ -deficient cultures quickly enhanced nitrogenase activity, in a much shorter time (2 h) than that required ( $\approx 20$  h) for the induction of nitrogenase synthesis. Moreover, the activity increased very rapidly and was fully restored 5–6 h after addition of  $\text{Na}^+$ . The minimum level of  $\text{Na}^+$  required for restoration of nitrogenase activity was calculated to be 25  $\mu\text{M}$  in *A. torulosa* (figure 1). These data suggested a role for  $\text{Na}^+$  in the activation of presynthesised enzyme rather than in its *de novo* synthesis. Attempts to demonstrate this by preventing fresh synthesis with chloramphenicol were however unsuccessful, since chloramphenicol was found to rapidly inhibit nitrogenase activity (Apte and Thomas, 1980).

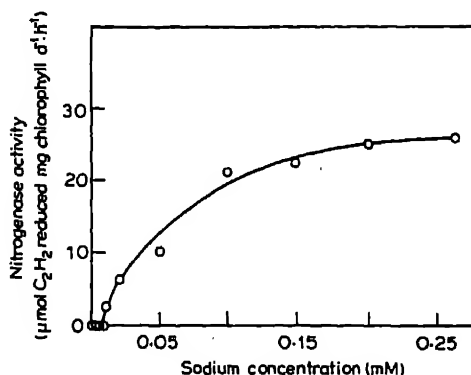


Figure 1. Determination of minimum level of external  $\text{Na}^+$  required for *in vitro* nitrogenase activity in *Anabaena torulosa*. Cyanobacterium was grown, in five-fold diluted combined nitrogen-free cyanophycean medium (CM/5; David and Thomas, 1979) devoid of  $\text{Na}^+$ , for 30 h. Different concentrations of  $\text{Na}^+$  were then added to separate aliquots of this culture and incubated under normal growth conditions. Nitrogenase activity ( $\text{C}_2\text{H}_2$  reduction) was determined 5 h after addition of  $\text{Na}^+$ , by the procedure of David *et al.* (1980).

A requirement of  $\text{Na}^+$  for nitrogenase activity has also been shown subsequently for the nonheterocystous cyanobacterium *Plectonema boryanum*-594 (Apte and Thomas 1984) which fixes  $\text{N}_2$  only under microaerophilic conditions. Comparisons were made between the electrophoretograms of sodium dodecyl sulphate (SDS) extracts of proteins obtained from  $\text{N}_2$ -fixing cultures with those repressed in the synthesis of nitrogenase. Using the approach along with the calibration of molecular weights, presumptive nitrogenase proteins having molecular weight of 63 K (MoFe protein) and 33 K (Fe protein) were detected in both  $\text{Na}^+$ -deficient as well as  $\text{Na}^+$ -supplemented *P. boryanum* (Apte and Thomas, 1984).

Recently we have obtained conclusive evidence for the non-requirement of  $\text{Na}^+$  for nitrogenase synthesis in *A. torulosa* using the approach described by Peterson and Wolk (1978). *A. torulosa* iron-containing proteins were radiolabelled with  $^{59}\text{Fe}$ . Soluble proteins were then extracted and electrophoresed on polyacrylamide gels under nondenaturing anaerobic conditions and the distribution of radioactivity was determined. In cultures without combined nitrogen and thus derepressed for nitrogenase, six  $^{59}\text{Fe}$  peaks were detected which corresponded with distinct protein bands. Of these, two iron-containing proteins absent in  $\text{NH}_4^+$ -grown repressed cultures and in extracts exposed to  $\text{O}_2$ , were identified as nitrogenase proteins. Both the proteins were present, in comparable amounts on unit protein basis, in derepressed cultures grown in the absence as well as in the presence of  $\text{Na}^+$  (table 1). It is thus clear that cyanobacterial nitrogenase proteins are synthesised even in the absence of  $\text{Na}^+$  but seem to function only in its presence. The cation thus appears to be essential for the expression of nitrogenase activity.

#### *Sodium, molybdenum and cyanobacterial nitrogenase*

A comparative analysis of  $\text{Na}^+$  and molybdenum requirements for nitrogen fixation has helped in obtaining more information on the role of  $\text{Na}^+$  (Apte and Thomas, 1984).

**Table 1.** Effect of sodium on the synthesis of nitrogenase proteins in *Anabaena torulosa*.

Culture conditions	Distribution of radioactivity (dpm mg total protein <sup>-1</sup> )	
	MoFe protein	Fe protein
Combined nitrogen-free CM/5*, devoid of Na <sup>+</sup>	60250	30100
Combined nitrogen-free CM/5*, with 1 mM Na <sup>+</sup>	66117	32604
CM/5* supplemented with 3 mM NH <sub>4</sub> Cl.	1429	0

Cyanobacterium was grown in culture media supplemented with radioactive <sup>59</sup>FeCl<sub>3</sub> (1 μCi . ml<sup>-1</sup>) for 30 h. Total proteins were extracted and electrophoresed on polyacrylamide vertical slab gels under nondenaturing, anaerobic conditions as described by Peterson and Wolk (1978). Gels were cut into 1 mm slices which were analysed for radioactivity by a NaI-crystal scintillation γ-spectrometer. Nitrogenase proteins were identified from among six <sup>59</sup>Fe-labelled iron proteins by: (i) incorporation of <sup>59</sup>Fe in them, (ii) their absence in NH<sub>4</sub><sup>+</sup>-grown cultures, (iii) their disappearance upon exposure to oxygen, (iv) their apparent molecular weights, and (v) rate of migration towards anode.

\* Five-fold diluted cyanophycean medium (David and Thomas, 1979).

Molybdenum forms a part of the iron molybdenum cofactor (FeMoCo) (Shah and Brill, 1977; Eady *et al.*, 1980), a constituent of the MoFe protein or component I of nitrogenase (Eady and Postgate, 1974) and is generally considered to be the substrate-binding active site of nitrogenase (Nagatani and Brill, 1974; Smith, 1977; Thorneley *et al.*, 1980). Molybdenum has also been implicated in the regulation of nitrogenase synthesis (Brill *et al.*, 1974; Kennedy and Postgate, 1977). This regulatory role has been a subject of controversy. Thus molybdenum is reported to be essential for the synthesis of both components I and II in *Clostridium pasteurianum* (Cardenas and Mortenson, 1975) while in *Klebsiella pneumoniae* (Kahn *et al.*, 1982) and *Azotobacter* (Nagatani and Brill, 1974; Pienkos *et al.*, 1981) inactive component I and active component II are synthesised in the absence of molybdenum. Although molybdenum is essential for cyanobacterial N<sub>2</sub> fixation (Wolfe, 1954 a, b; Taha and Elrefai, 1962; Fay and de Vasconcelos, 1974) it is not required for the synthesis of nitrogenase protein in *P. boryanum* (Nagatani and Haselkorn, 1978) and *A. cylindrica* (Hallenbeck and Benemann, 1980).

Many responses of N<sub>2</sub>-fixing cyanobacteria to Na<sup>+</sup> deficiency (Apte and Thomas, 1980, 1984) are similar to that observed during molybdenum deficiency (Wolfe, 1954a; Fay and de Vasconcelos, 1974; Apte and Thomas, 1984). Nitrogenase proteins are synthesised under both Na<sup>+</sup> (Apte and Thomas, 1984; and unpublished results) as well as molybdenum starvation (Nagatani and Haselkorn, 1978). Absence of either of the elements, however, results in loss of nitrogenase activity and restricts diazotrophic growth. In *A. torulosa*, addition of Na<sup>+</sup> as well as molybdenum to cultures deficient in the respective cation restores nitrogenase activity. The kinetics of the restoration

process, comprising a 2–3 h lag, subsequent to rapid increase and full restoration by 6 h is quite comparable for both  $\text{Na}^+$  and molybdenum (Apte and Thomas, 1984). In the nonheterocystous *P. boryanum*, enhancement of nitrogenase activity following addition of  $\text{Na}^+$  (Apte and Thomas, 1984) or molybdenum (Nagatani and Haselkorn, 1978) occurs much faster, thus indicating that in *Anabaena* a possible lag occurs in the transport of these ions into heterocysts.

Uptake of both molybdate (Wolfe 1954a, b) and  $\text{Na}^+$  (S. K. Apte and Joseph Thomas, unpublished results) is much higher under  $\text{N}_2$ -fixing conditions than in  $\text{NH}_4^+$ -grown cultures where synthesis of both nitrogenase and nitrate reductase is repressed. Nitrate-grown cultures also show a drastically reduced uptake of  $\text{Na}^+$  but uptake of molybdenum is substantial, being 50% of  $\text{N}_2$ -fixing cultures (S. K. Apte and Joseph Thomas, unpublished results). A molybdenum co-factor is a constituent of nitrate reductase, and a substantial uptake of molybdenum is probably needed to maintain nitrate reductase activity.

Molybdenum-deficient cultures of cyanobacteria differ from  $\text{Na}^+$ -deficient cultures in one important respect. While the absence of molybdenum enhances heterocyst differentiation (Fay and de Vasconcelos, 1974) it is unaffected by  $\text{Na}^+$  deficiency (Apte and Thomas, 1980). In cyanobacteria the heterocyst pattern is thought to be governed by a specific nitrogenous inhibitor, produced by preexisting heterocysts, which diffuses through the vegetative cells establishing decreasing concentration gradient away from heterocysts. New heterocysts arise by differentiation of those vegetative cells where the concentration of this inhibitor falls below a critical level (Fogg, 1949; Wolk, 1967; Wilcox *et al.*, 1973; Reddy and Talpasayi, 1974). Molybdenum deficiency results in nitrogen starvation apparently leading to a fall in the level of the nitrogenous heterocyst inhibitor. We find that  $\text{Na}^+$  deficiency also causes nitrogen starvation (Apte and Thomas, 1983a). The postulated inhibitor appears to be still present in  $\text{Na}^+$ -deficient cultures in quantities adequate to regulate the heterocyst pattern.

#### *Possible mechanism of nitrogenase activation by sodium*

Evidence for and against the possible ways by which the presence of  $\text{Na}^+$  may facilitate nitrogenase activity is considered below.

*Effect on heterocysts:* Since heterocysts are the sole sites of aerobic (Peterson and Burris, 1976; Thomas *et al.*, 1977; Peterson and Wolk, 1978) and possibly of even anaerobic (Murry *et al.*, 1984) nitrogen fixation in heterocystous cyanobacteria, a change in number or quality of heterocysts is bound to affect nitrogenase activity. However, as mentioned earlier,  $\text{Na}^+$  deficiency does not influence heterocyst differentiation (Apte and Thomas, 1980), nor are the heterocysts 'leaky' (structural or biochemical lesions) to  $\text{O}_2$  since even under anaerobiosis nitrogenase activity depends on  $\text{Na}^+$  (Apte and Thomas, 1982, 1984). Finally, the requirement is also observed in the nonheterocystous microaerophilic diazotroph, *Plectonema boryanum* (Apte and Thomas, 1984).

*Transport of molybdenum:*  $\text{Na}^+$  has been found to influence the transport of certain cations (Dewar and Barber, 1973), anions like phosphate (Kodama and Taniguchi, 1977) amino acids (Lanyi *et al.*, 1976; MacDonald *et al.*, 1977) and sugars (Stock and

Roseman, 1971) in bacteria. In view of the close resemblance of the effects of  $\text{Na}^+$  and molybdenum deficiency it was considered possible that  $\text{Na}^+$  deprivation might adversely affect molybdate transport. However, we have shown recently that in *A. torulosa* uptake of  $\text{Na}^+$  and molybdate is independent of the presence of the other ion both under  $\text{N}_2$ - and  $\text{NH}_4^+$ -supported growth.  $\text{Na}^+$ , even at concentrations 50 times higher than those of molybdenum, did not affect molybdate uptake (Apte and Thomas, 1984).

**Protection of nitrogenase from oxygen:** As mentioned earlier,  $\text{Na}^+$  dependence of cyanobacterial nitrogenase is also observed under anaerobic conditions (Apte and Thomas, 1984). Prolonged anaerobiosis enhances nitrogenase activity of  $\text{Na}^+$ -supplemented *A. torulosa* cultures by over threefold but does not affect the low activity of  $\text{Na}^+$ -deficient cultures. The dependence of nitrogenase activity of  $\text{Na}^+$  is thus not consequent to a role of the cation in protecting nitrogenase from oxygen.

**Effect on membrane potential and supply of reductant:** Nitrogenase needs a constant supply of reductant (electrons) for its activity. A possible route of electron transfer to cyanobacterial nitrogenase is from NADPH generated by oxidative pentose phosphate pathway via ferredoxin: NADP<sup>+</sup> oxidoreductase (Apte *et al.*, 1978; Lockau *et al.*, 1978). Such an electron transfer, considered to be thermodynamically unfavourable may become favourable under appropriate membrane potential *in vivo* (Haaker *et al.*, 1980). The importance of membrane potential in regulating nitrogenase activity has been demonstrated in several diazotrophs like *Azotobacter vinelandii* (Laane *et al.*, 1980), *Rhodospseudomonas capsulata* (Haaker *et al.*, 1982), *Rhizobium leguminosarum* bacteroids (Laane *et al.*, 1979) and *Anabaena variabilis* (Hawkesford *et al.*, 1981). In all these diazotrophs hyperpolarisation increases while depolarisation decreases nitrogenase activity. Paradoxically, in *A. torulosa*, while there is little or no nitrogenase activity during  $\text{Na}^+$  deficiency, a remarkable hyperpolarisation of membrane potential occurs, which is not reversed immediately by re-addition of  $\text{Na}^+$  (figure 2). A similar hyperpolarisation induced by the ionophore nigericin, however, enhances nitrogenase activity in  $\text{Na}^+$ -supplemented cultures (S. K. Apte and Joseph Thomas, unpublished results). Clearly, therefore, the effect of  $\text{Na}^+$  on nitrogenase activity is independent of its effect on membrane potential.

Possible influence of  $\text{Na}^+$  on generation and supply of electrons has not been examined so far. It will be interesting to see whether an externally supplied reductant like  $\text{Na}^+$ -free dithionite or more preferably a physiological source like glucose-6-phosphate stimulates nitrogenase activity in isolated heterocysts or cell-free extracts from  $\text{Na}^+$ -deficient cyanobacteria.

**Conformational activation:** Attempts have been made to ascertain whether  $\text{Na}^+$  deficiency directly or indirectly renders nitrogenase into inactive conformation and whether this can be reversed by preincubation under acetylene (Apte and Thomas, 1984). Preincubation under  $\text{C}_2\text{H}_2$  enhances *in vivo* nitrogenase activity of cyanobacteria (David *et al.*, 1978) by inducing conformational changes such that the affinity for several substrates is altered (Apte *et al.*, 1978). In purified nitrogenase proteins from *Klebsiella pneumoniae* (Thorneley and Eady, 1977) such treatment enhances electron flow through nitrogenase resulting in more and more reduced forms of enzyme and

enhancement of activities. On incubation with  $C_2H_2$ ,  $Na^+$ -supplemented *A. torulosa* shows over two-fold enhancement of  $C_2H_2$  reducing activity while the low activity of  $Na^+$ -deficient cultures is not affected (Apte and Thomas, 1984). Thus, a conformational activation by  $Na^+$ , if at all present, is different from that caused by exposure to  $C_2H_2$ . Possible direct effect(s) of  $Na^+$  on the enzyme molecule itself are not considered here in the absence of such data.

**Supply of ATP:**  $N_2$  reduction is a highly energy intensive process and nitrogenase consumes nearly 15 mol of ATP per mol of  $N_2$  reduced (Postgate, 1982).  $Na^+$  efficiency has been found to significantly decrease the uptake and utilisation of phosphate and thereby reduce the size of nucleotide pools. Restoration of nitrogenase activity following addition of  $Na^+$  to cultures deficient in the cation is preceded by significant enhancement of  $P_i$  uptake and of nucleotide levels (figure 2; and see also section on phosphate uptake and utilisation).

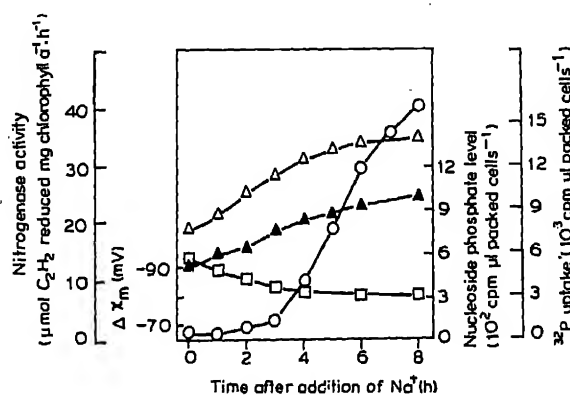


Figure 2. Effect of sodium on membrane potential ( $\square$ ), uptake of phosphate ( $\Delta$ ), nucleotide phosphate pool ( $\blacktriangle$ ) and nitrogenase activity ( $\circ$ ) of  $Na^+$ -deficient *Anabaena torulosa*. Membrane potential ( $\Delta\chi_m$ ) was determined by using lipophilic cation [ $^{14}C$ ]-tetraphenylphosphonium as described by Haaker *et al.* (1982). Uptake of  $^{32}P_i$  and its incorporation into nucleotide phosphates were determined by the procedures described by Sugino and Miyoshi (1964) and Kodama and Taniguchi (1977). Values of membrane potential (mV),  $P_i$  uptake and nucleotide phosphates (cpm  $\mu l$  packed cells $^{-1}$ ), and nitrogenase activity ( $\mu mol$   $C_2H_2$  reduced  $mg$  chlorophyll  $a^{-1} h^{-1}$ ) for corresponding  $Na^+$ -supplemented cultures at 0 h were -65.2, 19400, 1405 and 52.63 respectively.

Of all the possibilities discussed above, a limitation on the supply of available ATP to nitrogenase appears, at present, to be the best available explanation for loss of  $N_2$  fixation in the absence of  $Na^+$ . A role of  $Na^+$  in maintaining supply of ATP to support various energy-linked functions has also been observed in *Pseudomonas stutzeri* (Kodama and Taniguchi, 1976, 1977).

#### Sodium requirement for nitrate assimilation

Although a requirement of  $Na^+$  for nitrate-supplemented growth of both diazotrophic and diazotrophic cyanobacteria seems fairly established the nature of such

requirement is not very clear. In nitrate-supplemented *A. cylindrica*, Brownell and Nicholas (1967) found that  $\text{Na}^+$  deficiency resulted in several fold enhancement of nitrate reductase activity resulting in overproduction of nitrite which accumulated in the medium in toxic concentrations and caused loss of phycocyanin and chlorosis of the cultures. The  $\text{Na}^+$ -deficient cultures showed reduced dry wt and protein content but exhibited enhanced incorporation of  $^{15}\text{NO}_3$ ,  $^{15}\text{NO}_2$ ,  $^{15}\text{NH}_4^+$  and  $[^{14}\text{C}]$ -glutamate into proteins. Based on results obtained using chloramphenicol, it was concluded (Brownell and Nicholas, 1967) that  $\text{Na}^+$  was essential for the synthesis of a protein which regulated (decreased) nitrate reductase activity. No attempts were, however, made to detect such a regulatory protein in  $\text{Na}^+$ -supplemented cultures. Possible effects of  $\text{Na}^+$  on the uptake of nitrate and nitrite and on nitrite reduction were not examined. It is also not clear from the data why, inspite of enhanced nitrate reduction and incorporation into proteins, the cultures showed symptoms of nitrogen starvation such as decreased protein content and more importantly, loss of phycocyanin.

Nitrate-supplemented,  $\text{Na}^+$ -deficient cultures of a related cyanobacterium, *A. torulosa*, also showed loss of chlorophyll *a* but this was not due to accumulation of nitrite which was negligible (S. K. Apte and Joseph Thomas, unpublished results). As compared to  $\text{Na}^+$ -supplemented cultures,  $\text{Na}^+$ -deficient cultures showed reduced uptake of nitrite as has also been reported in a diatom *Phaeodactylum* (Cresswell and Syrett, 1982). In contrast to the results obtained with *A. cylindrica*, we found that in *A. torulosa* both *in vivo* as well as *in vitro* nitrate reductase activity was decreased by prolonged  $\text{Na}^+$  deficiency. The observed decrease in nitrate reduction (25–30%) was however insufficient to explain the extent of growth inhibition (50%) of  $\text{Na}^+$ -deficient nitrate-supplemented *A. torulosa* (S. K. Apte and Joseph Thomas, unpublished results).

It appears therefore that a requirement of  $\text{Na}^+$  for nitrate-supported growth of cyanobacteria may occur not only at the step of reduction of nitrate, or uptake and reduction of nitrite, but perhaps also at a later stage in the metabolism. More work is warranted to resolve the nature of  $\text{Na}^+$  requirement in nitrate-grown cyanobacteria.

#### *Synthesis of amino acids and proteins*

$\text{Na}^+$  deficiency results in a significant reduction (50–70%) in the content of total nitrogen and nitrogen based compounds, especially amino acids and proteins, in  $\text{N}_2$ -fixing *A. torulosa* cultures. In comparison, a similar treatment of nitrate- or  $\text{NH}_4^+$ -supplemented cultures causes only a 15–25% reduction (S. K. Apte and Joseph Thomas, unpublished results). However, the activities of two key enzymes involved in  $\text{NH}_4^+$  assimilation and synthesis of other amino acids, namely glutamine synthetase and aminotransferase, are independent of the presence of  $\text{Na}^+$ . Similarly the rate of amino acid incorporation into proteins is not reduced in  $\text{Na}^+$ -deficient cultures of *A. torulosa*. In fact, the rate of protein synthesis is enhanced under  $\text{Na}^+$  starvation. Thus, the reduced contents of amino acids and proteins result not from limitations on the efficiency of machinery responsible for their synthesis but indirectly from loss of nitrogenase activity and nitrogen starvation.

There is increased incorporation of glutamate into protein but decreased protein content in  $\text{Na}^+$ -deficient, nitrate-supplemented cultures of *A. cylindrica* (Brownell and Nicholas, 1967). The decreased protein content in this case also probably results from

nitrogen starvation caused by loss of nitrate reductase activity (see earlier part of this section). It appears that in cyanobacteria, as also in *E. coli* (Lubin and Ennis, 1964), presence of excess  $\text{Na}^+$  decreases the rate of protein synthesis probably by antagonising the established stimulatory effect of  $\text{K}^+$  on amino acid incorporation (Harold, 1982).

In addition to the quantitative differences,  $\text{Na}^+$  also seems to determine the quality of proteins synthesised by  $\text{N}_2$ -fixing *A. torulosa* cultures. Thus  $\text{Na}^+$ -deficient cultures lack two proteins characteristically present in  $\text{Na}^+$ -supplemented cultures; the role of these proteins is unknown at present.  $\text{Na}^+$ -deficient cultures also contain reduced amounts of phycocyanin—an established marker of nitrogen status in cyanobacteria (Van Gorkom and Donze, 1971; De Vasconcelos and Fay, 1974) and certain other proteins probably including some of the Calvin cycle enzymes (S. K. Apte and Joseph Thomas, unpublished data).

## Sodium requirement in general metabolism

### Photosynthesis

Under  $\text{N}_2$ -fixing conditions,  $\text{Na}^+$  deficiency causes impairment of photosynthetic functions (Apte and Thomas 1982, 1983a).  $\text{Na}^+$ -deficient *A. torulosa* cultures show loss of photosynthetic pigments and severe inhibition of photoevolution of  $\text{O}_2$  and photofixation of  $\text{CO}_2$ . The content of nitrogenous pigments (chlorophyll *a* and phycocyanin) decreases while that of carotenoids increases. Similar loss of pigments occurs also in  $\text{Na}$ -deficient *A. cylindrica* (Brownell and Nicholas, 1967). Phycocyanin is a major constituent of the oxygen-evolving apparatus of photo-system II but since the phycocyanin/chlorophyll *a* ratio is not affected by  $\text{Na}^+$  deficiency the overall pigment composition of photosystem II probably remains unaltered.  $\text{Na}^+$  deficiency also does not affect methyl viologen-induced and light-dependent oxygen uptake (Apte and Thomas, 1982, 1983a). Since this reaction collectively represents all the major photochemical reactions (photolysis of water, electron flow through the entire 'Z' scheme and electron donation to the terminal electron acceptor), the possibility of impairment of structural/functional integrity of photo-systems by  $\text{Na}$ -deficiency is unlikely.

Addition of  $\text{Na}^+$  (as  $\text{NaCl}$ ) to  $\text{Na}^+$ -deficient *A. torulosa* cultures restores photoevolution of  $\text{O}_2$  and  $\text{CO}_2$  fixation (Apte and Thomas, 1983a). This effect of  $\text{NaCl}$  is not simulated by several other chlorides indicating that it is a genuine  $\text{Na}^+$  effect and not the 'chloride effect' observed in certain chloroplast preparations (Hind *et al.*, 1969; Izawa *et al.*, 1969). Interestingly,  $\text{NH}_4\text{Cl}$ , glutamine and glutamate completely mimic the  $\text{Na}^+$  effect (table 2). Addition of these compounds to  $\text{Na}^+$ -deficient cultures also restores the photoevolution of  $\text{O}_2$  and  $\text{CO}_2$  fixation; glutamine and glutamate, in fact, are more efficient than  $\text{Na}^+$  (Apte and Thomas, 1983a).

It is well known that physiological photoevolution of  $\text{O}_2$  occurs in the presence of a terminal electron acceptor ( $\text{CO}_2$ ) which is reduced. The extremely poor rates of  $\text{CO}_2$  fixation in  $\text{Na}^+$ -deficient *A. torulosa* thus appear to be responsible for very low rates of  $\text{O}_2$  photoevolution (Apte and Thomas, 1983a). This is supported by the fact that provision of an alternative electron acceptor (like methyl viologen) facilitates normal reaction (table 2). Moreover, the reappearance of  $\text{O}_2$  photoevolution by addition of



**Table 2.** Impairment of photosynthesis by sodium deficiency and its restoration by addition of sodium or products of  $N_2$  fixation in diazotrophically grown *Anabaena torulosa*.

Culture conditions	Additions	Photosynthetic reactions ( $\mu\text{mol. mg chlorophyll } a^{-1} \text{ min.}^{-1}$ )		
		Photo-evolution of $O_2$	Methyl viologen- induced $O_2$ uptake in light	Light-dependent uptake of $NaH^{14}CO_3$
$Na^+$ -supplemented	Nil	9.86	7.15	3.51
$Na^+$ -deficient	Nil	3.51	7.70	1.23
$Na^+$ -deficient	$NaCl$ (1 mM)	8.06	7.06	2.05
$Na^+$ -deficient	$KCl$ (1 mM)	3.65	N.D.	N.D.
$Na^+$ -deficient	$NH_4Cl$ (3 mM)	5.24	7.38	N.D.
$Na^+$ -deficient	Glutamine (1 mM)	8.35	6.42	N.D.
$Na^+$ -deficient	Glutamate (1 mM)	10.06	8.30	2.46

Thirty h after transfer to  $Na^+$ -free media, cyanobacterium was harvested and resuspended in identical medium. Additions were then made as shown and samples were incubated for 2 h under aeration ( $2 \text{ l. min}^{-1}$ ) at 5000 lx and  $25^\circ\text{C}$  prior to analysis. Photosynthetic reactions were studied as described by Apte and Thomas (1983a).

N.D. = not determined.

$Na^+$  or amino acids is accompanied by a concomitant restoration of  $CO_2$  fixation (Apte and Thomas, 1983a). Loss of  $CO_2$  fixation appears to be related to nitrogen starvation which occurs under  $Na^+$  deficiency. Nitrogen starvation decreases protein synthesis and causes degradation of proteins in *Anabaena* (Ownby *et al.*, 1979) probably due to activation of certain proteases (Wood and Haselkorn, 1977).  $Na^+$ -deficient cultures also show loss of and decreased content of certain proteins (see section on nitrogen assimilation) and if some of these include Calvin cycle enzymes it may explain the reduced  $CO_2$  fixation under  $Na^+$  deficiency.

To summarise, it can be concluded that  $Na^+$  deficiency influences photosynthesis indirectly through nitrogen starvation: (i) in media supplemented with combined nitrogen (especially  $NH_4^+$ )  $Na^+$ -deficiency does not affect photosynthesis; (ii)  $Na^+$ -deficiency results in loss of nitrogenase activity; (iii) nitrogen starvation is evident from the decreased content of total nitrogen, proteins, amino acids and nitrogenous pigments in  $Na^+$ -deficient cultures; (iv) nitrogen starvation has been found to decrease photosynthetic  $CO_2$  fixation in the cyanobacterium *A. cylindrica* (Lawrie *et al.*, 1976); (v) inhibition of photosynthesis is reversed upon addition of  $Na^+$  which 'switches on' nitrogenase activity; (vi)  $NH_4^+$ , glutamine and glutamate, which are the initial products of  $N_2$  fixation and assimilation in cyanobacteria (Thomas *et al.*, 1975), also rapidly restore photosynthesis in  $Na^+$ -deficient *A. torulosa*. The greater efficiency of amino acids, compared to  $Na^+$ , is probably because they by-pass the energy-consuming nitrogenase reaction.

### Respiration and oxidative phosphorylation

In the unicellular cyanobacterium, *Anacystis nidulans*, high concentrations of  $\text{Na}^+$  have been found to stimulate respiratory  $\text{O}_2$  consumption (Nitschmann and Peschek, 1982; Nitschmann *et al.*, 1982) and anaerobically grown cells were seen to accumulate much more  $\text{Na}^+$  than aerobically maintained cells. These results suggested that  $\text{Na}^+$  extrusion by  $\text{Na}^+/\text{H}^+$  antiporter is coupled to the respiratory proton gradient and that part of the energy stored in the proton gradient would be dissipated by  $\text{Na}^+$  extrusion decreasing the efficiency of oxidative phosphorylation. A fall in the P/O ratio from 2.9 at low  $\text{Na}^+$  concentration to 0.6 at high concentration (Nitschmann and Peschek, 1982) and decrease of ATP pools in the presence of high  $\text{Na}^+$  (Nitschmann *et al.*, 1982) substantiated the above conclusion. Such deleterious effect of  $\text{Na}^+$  was not found in two facultative chemoheterotrophic diazotrophs *Anabaena variabilis* and *Nostoc* MAC. It has been, therefore, proposed that the high respiratory energy costs of  $\text{Na}^+$  extrusion are responsible for the obligate photoautotrophy of most cyanobacteria.

In  $\text{N}_2$ -fixing *A. torulosa* addition of 10–100 mM NaCl has no effect on respiratory  $\text{O}_2$  consumption (S. K. Apte and Joseph Thomas, unpublished results). This resembles the situation reported in *A. variabilis* and *Nostoc* MAC (Nitschmann *et al.*, 1982). Since all these cyanobacteria are diazotrophs requiring  $\text{Na}^+$ , it is tempting to suggest that the energy expensive  $\text{Na}^+$  extrusion in *A. nidulans* may be associated, in addition to obligate photoautotrophy, with adiazotrophy also. As we shall see later (section on sodium transport)  $\text{Na}^+$  extrusion in certain diazotrophic cyanobacteria does not seem to be mediated by a  $\text{Na}^+/\text{H}^+$  antiporter and this may be a useful device to economise and divert the available energy to a more useful process like  $\text{N}_2$  fixation.

Effect of  $\text{Na}^+$  deficiency on respiration has been examined in *A. torulosa* (Apte and Thomas, 1982) where it increases the dark consumption of  $\text{O}_2$ .  $\text{Na}^+$  deficiency also decreases the uptake of  $P_i$  (figure 2) and together this should result in low P/O ratios and decreased efficiencies of oxidative phosphorylation (S. K. Apte and Joseph Thomas, unpublished results).

### Uptake and utilisation of phosphate

$\text{Na}^+$  deficiency curtails the uptake and utilisation of phosphate in *A. torulosa* grown under  $\text{N}_2$ -fixing conditions and results in a marked depletion of nucleotide pool (mostly nucleotide triphosphates). Addition of  $\text{Na}^+$  to  $\text{Na}^+$ -deficient *A. torulosa* cultures quickly enhances both the uptake of phosphate and nucleotide levels (figure 2). Similar effects of  $\text{Na}^+$  on phosphate uptake and ATP synthesis occur in *Pseudomonas stutzeri* (Kodama and Taniguchi, 1976, 1977). In *A. torulosa* the restoration of these processes precedes that of nitrogenase activity. A detailed investigation of influence of  $\text{Na}^+$  on phosphate uptake systems in  $\text{N}_2$ -fixing cyanobacteria is necessary to identify the nature of regulation of  $P_i$  transport by  $\text{Na}^+$ . It also needs to be determined whether regulation of  $P_i$  uptake by  $\text{Na}^+$  is characteristic of diazotrophic growth of cyanobacteria (which depends on  $\text{Na}^+$ ) alone or also occurs during adiazotrophic growth.

### Transport of sugars and amino acids

Certain halophilic (Lanyi *et al.*, 1976) and alkalophilic (Koyama *et al.*, 1976) bacteria couple  $\text{Na}^+$  gradient with symport/antiport of sugars and amino acids. A requirement

of  $\text{Na}^+$  for transport of glucose and glutamate is also observed in other bacteria, e.g., *Escherichia coli* (Frank and Hopkins, 1969; Halpern *et al.*, 1973), *Salmonella typhimurium* (Stock and Roseman, 1971), *Pseudomonas stutzeri* (Kodama and Taniguchi, 1977). In a recent investigation (Apte, 1984) we found that absence of  $\text{Na}^+$  did not drastically affect the uptake of ( $^{14}\text{C}$ ) glutamate and ( $^{14}\text{C}$ ) glucose in  $\text{N}_2$ -fixing cultures of *A. torulosa*. However, low concentrations (1–5 mM) of  $\text{Na}^+$  stimulated uptake of these solutes while higher concentrations inhibited glutamate uptake probably by lowering the membrane potential. Similar effect of  $\text{Na}^+$  on glutamate transport has also been reported in *A. nidulans* (Lee-Kaden and Simonis, 1982).

### Sodium transport and salt tolerance of cyanobacteria

#### Sodium transport

Studies on ion transport, in general, have received only scant attention in cyanobacteria. In view of the salt tolerance and the requirement of  $\text{Na}^+$  for growth exhibited by cyanobacteria, studies on the transport of  $\text{Na}^+$  are essential prerequisites for understanding sodium metabolism of these microbes. Such studies are also considered advantageous for understanding the mechanism of salt tolerance of higher plants (Szalay and MacDonald, 1980). In spite of this  $\text{Na}^+$  transport has been examined in detail only in the unicellular diazotroph *A. nidulans* (Dewar and Barber, 1973; Paschinger, 1977; Nitschmann *et al.*, 1982) and in filamentous  $\text{N}_2$ -fixing species of *Anabaena* (Apte and Thomas, 1983b).

The influx of  $\text{Na}^+$  occurs by passive diffusion in *A. nidulans* (Dewar and Barber, 1973; Paschinger, 1977). In two *Anabaena* spp. (Apte and Thomas, 1983b)  $\text{Na}^+$  influx has been shown to be carrier-mediated and follows Michaelis-Menten kinetics. The affinity ( $K_m$ ) of the carrier for  $\text{Na}^+$  is different in the fresh water cyanobacterium *Anabaena* L-31 (2.8 mM) from that of the brackish water species *A. torulosa* (0.3 mM), probably in accordance with their metabolic requirement of  $\text{Na}^+$ . The modulation of  $\text{Na}^+$  influx in *Anabaena* spp. by concanavalin A, known for its ability to bind and reorient certain sugar moieties, suggests that the  $\text{Na}^+$  carrier responsible for influx may be a glycoprotein (Apte and Thomas, 1983b).

$\text{Na}^+$  influx in *Anabaena* spp. has been found to be mediated by a secondary active transport which occurs in response to the proton-motive force generated by the primary active extrusion of protons (S. K. Apte and Joseph Thomas, unpublished results). Of the two components that constitute the proton electrochemical potential gradient, namely a pH gradient and membrane potential,  $\text{Na}^+$  influx is more closely linked with the membrane potential. Nigericin, an ionophore known for its ability to collapse the pH gradient and to convert it into membrane potential at pH 6.0 (Haaker *et al.*, 1980; Hawkesford *et al.*, 1981), causes significant hyperpolarisation of membrane potential in *Anabaena* spp. This is accompanied by concomitant increase in  $\text{Na}^+$  influx, thus clearly establishing the regulation of  $\text{Na}^+$  influx by membrane potential (S. K. Apte and Joseph Thomas, unpublished results). In conformity with this, all treatments which cause depolarisation of membrane such as low temperature, dark incubation, inhibition of respiration by cyanide, azide and anaerobiosis, and ATPase inhibitors [e.g., carbonyl-cyanide m-chlorophenyl-hydrazone (CCCP) and  $\text{N,N'}$ -dicyclohexyl-

carbodiimide (DCCD)] inhibit  $\text{Na}^+$  influx (figure 3). Similarly, hyperpolarisation caused by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,4-dinitrophenol (DNP) or nigericin is accompanied by enhanced  $\text{Na}^+$  influx (S. K. Apte and Joseph Thomas, unpublished results). The transition from an external pH of 6.0 to 8.0 severely inhibits  $\text{Na}^+$  influx in *Anabaena* spp. possibly due to reduced composite proton-motive force at higher pH as has been observed in *A. variabilis* (Reed *et al.*, 1980).

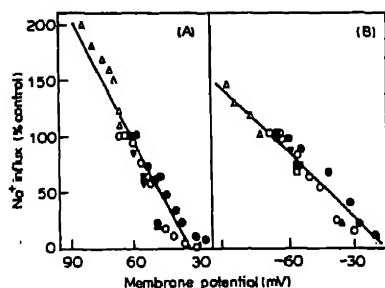


Figure 3. Regulation of sodium influx by membrane potential in nitrogen-fixing cyanobacteria: (A) *Anabaena torulosa* (B) *Anabaena* L-31. Data were pooled from experiments using CCCP, (O); DCCD, (●); nigericin, (Δ); low temperature (4°C), (▲); anaerobiosis (argon) (▽); dark incubation, (▼); cyanide, (□); and azide, (■).

$\text{Na}^+$  influx in *Anabaena* spp. has been found to be insensitive to low concentrations (1–5 mM) of external  $\text{K}^+$  (S. K. Apte and Joseph Thomas, unpublished results). Higher concentrations of  $\text{K}^+$  (10–100 mM) do inhibit  $\text{Na}^+$  influx but the calculated  $K_m$  values (25–50 mM) are far beyond the known levels of  $\text{K}^+$  in various eco-systems and therefore are probably of no eco-physiological significance. High selectivity for  $\text{K}^+$  and the ability to discriminate against  $\text{Na}^+$  is a general feature of cation transport in bacteria (Harold and Papineau, 1972; Rothstein, 1972), algae (Schaeble and Jacobson, 1967; Shieh and Barber, 1971) and higher plants (Rains, 1972; Flowers *et al.*, 1977; Epstein, 1980). The unicellular cyanobacterium *A. nidulans* also shows selective uptake of  $\text{K}^+$  against  $\text{Na}^+$  (Dewar and Barber, 1973). As against these, *Anabaena* spp. appear to transport  $\text{Na}^+$  and  $\text{K}^+$  independently. Indeed, in *A. variabilis*  $\text{K}^+$  transport occurs by  $\text{K}^+/\text{K}^+$  exchange diffusion which does not catalyse  $\text{Na}^+/\text{K}^+$  exchange (Reed *et al.*, 1981). The relationship between lack of selectivity between  $\text{Na}^+$  and  $\text{K}^+$  and the requirement of  $\text{Na}^+$  in *Anabaena* spp., if any, remains to be established. Increasing external concentrations of both  $\text{Na}^+$  or  $\text{K}^+$  does not significantly affect the membrane potential although some depolarisation occurs above 60 mM (S. K. Apte and Joseph Thomas, unpublished results). It is unlikely, therefore, that membrane potential may be constituted of a significant diffusion potential of  $\text{Na}^+$  or  $\text{K}^+$  ions in cyanobacteria.

Both *A. nidulans* (Paschinger, 1977) and *Anabaena* spp. (Apte and Thomas, 1983b) maintain a low internal  $\text{Na}^+$  concentration brought about by an active extrusion of the cation. The plasmalemma of *A. nidulans* is equipped with a DCCD-sensitive,  $\text{H}^+$ -translocating ATPase (Paschinger, 1977) and an  $aa_3$  type of  $\text{H}^+$ -extruding terminal cytochrome oxidase (Nitschmann and Peschek, 1982; Nitschmann *et al.*, 1982). The resulting proton gradient is coupled with a  $\text{Na}^+/\text{H}^+$  antiporter which brings about an

active extrusion of  $\text{Na}^+$ . When subjected to anaerobiosis or incubated with DCCD, therefore, *A. nidulans* shows net accumulation of  $\text{Na}^+$  (Paschinger, 1977; Nitschmann and Peschek, 1982). In *A. variabilis* proton efflux appears to be mediated by ATPase rather than by respiratory electron transport (Scherer *et al.*, 1984). We have obtained evidence recently that two *Anabaena* spp. generate proton gradients by means of extrusion of  $\text{H}^+$  carried out by both DCCD-sensitive ATPase(s) and respiratory oxidase(s) located in the plasmalemma. These cyanobacteria also exhibit rapid, active efflux of  $\text{Na}^+$  but this is not mediated by  $\text{Na}^+/\text{H}^+$  antiporter since DCCD treatment or anaerobiosis does not cause net  $\text{Na}^+$  accumulation (Apte, 1984). The identical sensitivity of both influx/efflux to *trans* concentrations of  $\text{Na}^+$ , to low temperatures ( $4^\circ\text{C}$ ) and to combined nitrogen suggests that the same carrier may facilitate both inward and outward  $\text{Na}^+$  fluxes.

Recently we have also identified an interesting regulation of  $\text{Na}^+$  transport by combined nitrogen in two *Anabaena* species. (S. K. Apte and Joseph Thomas, unpublished results). When grown in the presence of nitrate or  $\text{NH}_4^+$ , *Anabaena* L-31 and *A. torulosa* showed depolarisation of membrane potential and inhibition of  $\text{Na}^+$  influx compared to cultures grown in the absence of combined nitrogen. This was due to a direct effect of combined nitrogen on membrane potential and  $\text{Na}^+$  influx since addition of nitrate or  $\text{NH}_4^+$  to  $\text{N}_2$ -fixing cultures, only 2 min prior to assay, also produced identical effects. Addition of nitrate or  $\text{NH}_4^+$  *per se* also caused extrusion of  $\text{Na}^+$  preabsorbed by  $\text{N}_2$ -fixing cultures of *Anabaena* spp.. Together, the effect of combined nitrogen on influx as well as efflux processes allowed very little  $\text{Na}^+$  to accumulate in the cells. Even at 100 mM external NaCl concentration, nitrate and  $\text{NH}_4^+$  decreased the intracellular  $\text{Na}^+$  levels by 68 % and 50 % in *Anabaena* L-31 and by 45 and 40 % in *A. torulosa*. Thus, *Anabaena* spp. seem to have evolved an attractive mechanism mediated by combined nitrogen to curtail the entry and accumulation of  $\text{Na}^+$ , an ion not required in large quantity under nitrogen-supplemented conditions. The precise mechanism of this regulation awaits analysis.

#### *Cellular distribution of absorbed sodium and its incorporation into biomolecules*

Using the radiotracer  $^{22}\text{Na}^+$ , over 90 % of the total cell-bound  $\text{Na}^+$  of  $\text{N}_2$ -fixing cultures of *A. torulosa* was found to exist extracellularly in a freely exchangeable state; most of it was held by the extracellular mucopolysaccharide sheath of the cyanobacterium (S. K. Apte and Joseph Thomas, unpublished results). Nearly all of the intracellular  $\text{Na}^+$  (> 96 %) occurred as free  $\text{Na}^+$  in an osmotically active state. There was no evidence for binding or incorporation of  $\text{Na}^+$  into any of the biochemical fractions examined, especially proteins or carbohydrates, in *A. torulosa* grown on  $\text{N}_2$ , nitrate or  $\text{NH}_4^+$ . In *Anabaena*  $\text{Na}^+$  is, therefore, not a constituent of a storage product like the  $\text{Na}^+$ -mannoglycerate in certain red algae (Craigie, 1974). Also, no  $\text{Na}^+$ -binding or -storing protein, similar to molybdenum storage protein of certain diazotrophs (Pienkos and Brill, 1981), could be autoradiographically detected in *A. torulosa* both under repressed as well as induced conditions of nitrogenase synthesis. Thus, unlike molybdenum,  $\text{Na}^+$  appears to regulate nitrogenase activity and cyanobacterial metabolism as a cation, *per se*.

*Physiological basis of salt tolerance*

Many cyanobacteria exhibit considerable tolerance to salt (NaCl) and occurrence of marine, salt lake-inhabiting or brackish water forms is by no means rare (Desikachary, 1959; Fogg, 1973). There are also well documented cases of halophilic cyanobacteria, such as *Microcoleus chthonoplastes* (20–25% NaCl; Van Baalen, 1962), *Spirulina subsalsa* (> 3 M NaCl; Fogg, 1973), *Aphanothece halophytica* (> 3 M NaCl; Yopp *et al.*, 1978) and *Calothrix scopulorum* (5% NaCl; Stewart, 1964; Tel-Or, 1980a) and euryhaline cyanobacteria, capable of growth in freshwater and in varying degrees of salinity (Richardson *et al.*, 1983; Reed and Stewart, 1983). The physiological basis of their salt tolerance has been studied to some extent but remains not fully understood. Following types of mechanisms have been reported:

- (i) Accumulation of  $K^+$  occurs in response to increasing external salt concentration and helps in osmoregulation in *Aphanothece halophytica* (Miller *et al.*, 1976; Yopp *et al.*, 1978). Although this organism also accumulates carbohydrates and amino acids as osmotica these are considered secondary events triggered by high intracellular  $K^+$  concentration.
- (ii) Exclusion of  $Na^+$  and maintenance of low intracellular  $Na^+$  concentrations appear to be responsible for the salt tolerance of  $N_2$ -fixing cultures of *A. torulosa*, a brackish water species (Apte and Thomas, 1983b). As compared to the fresh water species *Anabaena* L-31, *A. torulosa* shows much reduced influx of  $Na^+$ , much higher affinity of carrier for  $Na^+$  which prevents excess influx at high  $Na^+$  concentrations, and more efficient efflux of  $Na^+$  resulting in lower internal  $Na^+$  concentrations. As mentioned earlier, presence of nitrate or  $NH_4^+$  severely curtailed influx and stimulated efflux of  $Na^+$  in both these *Anabaena* spp. As a consequence, salt tolerance of the fresh water and brackish water species was comparable and was 3–5 fold greater than that of corresponding  $N_2$ -fixing cultures (table 3).

**Table 3.** Comparative halotolerance of a brackish water and a freshwater species of *Anabaena* grown on different nitrogen sources.

Cyanobacterium	Nitrogen source during growth	Growth ( $\mu g$ chlorophyll <i>a</i> ) on NaCl (mM)					
		1	35	85	125	170	250
<i>Anabaena torulosa</i>	$N_2$	7.5 (7.3)	12.6 (9.1)	6.2 (9.2)	4.4 (8.1)	2.3 (5.4)	2.1 (3.6)
	$(NO_3)^{2-}$ (10 mM)	5.9	6.6	6.8	6.3	6.0	5.3
	$NH_4^+$ (3 mM)	9.4	12.1	14.1	12.6	8.5	5.9
<i>Anabaena</i> L-31	$N_2$	7.9 (4.6)	3.6 (1.0)	2.1 (0.3)	0.6 (0.1)	0.3 (0.0)	0.2 (0.0)
	$(NO_3)^{2-}$ (10 mM)	6.1	5.9	5.5	5.5	5.2	4.8
	$NH_4^+$ (3 mM)	10.3	13.2	9.3	8.0	7.8	7.5

*Anabaena torulosa* (a brackish water species) and *Anabaena* L-31 (a freshwater species) were grown on solid media (1% agar in CM/5) supplemented with or without combined nitrogen under continuous illumination (5000 lx). Representative data obtained (six replicates each) on fifth day after inoculation are presented. Chlorophyll *a* content was determined after Mackinney (1941). Values in parantheses show the nitrogenase activities in  $\mu mol$   $C_2H_4$  reduced  $\cdot mg$  chlorophyll  $a^{-1} \cdot h^{-1}$ .

(iii) Synthesis of internal osmotica in the form of carbohydrates, polyols, amino acids and quaternary amines facilitates osmoregulation in cyanobacteria. Two to four fold enhanced synthesis of carbohydrates, polyols and amino acids has been reported in *A. halophytica* (Yopp *et al.*, 1978). The main low molecular weight carbohydrate in *Rivularia atra* incubated in 100% sea water is the disaccharide trehalose (Reed and Stewart, 1983). It has been suggested that marine forms synthesise a unique compound glucosylglycerol (Mackay *et al.*, 1983) for osmoregulation while sucrose is characteristic of fresh water forms (Blumwald *et al.*, 1983). Richardson *et al.* (1983) however showed that *Synechocystis* PCC 6803 isolated from fresh waters also synthesised glucosylglycerol. In a recent study Reed *et al.* (1984) examined 70 cyanobacterial strains for the presence of these compounds and showed that although there is a trend for accumulation of glucosylglycerol in marine species and sucrose in fresh water species, no absolute link exists between a genus or an ecological type on the one hand and the chemical nature of carbohydrate accumulated on the other.

In *Anabaena* spp. we have observed an increased nitrogen demand during salt stress. Under  $N_2$ -fixing condition this is exemplified by the salt-tolerant *A. torulosa* which responds to salinity (upto 85 mM NaCl) by enhanced nitrogenase activity while the salt-sensitive *Anabaena* L-31 fails to respond in this manner. The enhanced salt tolerance of these species in the presence of combined nitrogen (table 3) is also in agreement with this. Thus, nitrogen supplementation appears to subsidise the increased nitrogen demand and hence result in increased tolerance to salt. Such increased demand is probably aimed at synthesis of nitrogenous osmoregulatory compounds like amino acids (Measures, 1975) and quaternary ammonium compounds (Shkedy-Vinkler and Avi-Dor, 1975; Galinsky and Truper, 1982). Indeed, the role of glycinebetaine and other quaternary ammonium compounds in osmoregulation has been demonstrated in the halophilic *Synechocystis* DUN52 (Mohammad *et al.*, 1983).

(iv) Cyanobacterial metabolism adapts to salt stress and certain metabolic features likely to be important in salt tolerance have been identified. Although, high NaCl concentrations inhibit photosynthesis and decrease the level of pigments, fatty acids and hydrocarbons in cyanobacteria (Batterton and Van Baalen, 1971), in a comparative study of four cyanobacteria Tel-Or (1980a,b) found that photosynthesis (photoevolution of  $O_2$ ) was more resistant to salt stress than  $N_2$  fixation. The sensitivity of nitrogenase activity was found to be consequent to the very high sensitivity of ferredoxin-NADP<sup>+</sup>-oxidoreductase to salt. The activity of this enzyme, which has been shown to mediate electron flow to cyanobacterial nitrogenase (Apte *et al.*, 1978; Lockau *et al.*, 1978) had a positive correlation with the degree of salt tolerance of different cyanobacteria. The activities of glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and photosystem I-mediated electron flow, all of which support nitrogenase activity, showed less sensitivity to salt while glutamine synthetase was almost insensitive (Tel-Or, 1980a,b). Among *Anabaena* spp., the salt-tolerant *A. torulosa* shows greater resistance (50% inhibition at 180 mM NaCl) of nitrogenase activity to salt than the salt-sensitive (50% inhibition at 25 mM NaCl) *Anabaena* L-31 (Apte and Thomas, 1983b). In *A. torulosa* the NaCl concentration responsible for 50% inhibition of growth (115 mM) and  $N_2$  fixation (> 180 mM) differ widely. Thus the growth inhibition in cyanobacteria under salt stress does not appear to be a direct consequence of inhibition of  $N_2$  fixation (S. K. Apte and Joseph Thomas, unpublished results).

*Reclamation of saline/alkali/sodic soils by cyanobacteria*

Salt tolerance of cyanobacteria has been exploited in the reclamation of agricultural habitats rendered useless due to excess salt. Singh (1950, 1961) reported that alkali soils of Northern India, variously called reh, kallar or usar, could be recalcimed through naturally occurring population of cyanobacteria, for subsequent cultivation of rice and sugarcane. After substantial growth had occurred during rainy season cyanobacterial mats were ploughed in and left buried in the soil. The annual addition of organic carbon was 35–60% and that of nitrogen 30–40% over the initial levels. After the first year the pH of soil fell from 9.5 to 7.6 and there was improvement in tilth, exchangeable calcium (20–33%) and water holding capacity (40%). It has been claimed that cyanobacterial incorporation into soil in this manner conserves organic carbon and nitrogen and immobilises  $\text{Na}^+$ , converting the sodium clay into a calcium type (Singh, 1950, 1961). However, as mentioned earlier, in aerated laboratory cultures, almost all of the absorbed  $\text{Na}^+$  remains osmotically active (S. K. Apte and Joseph Thomas, unpublished results). It is therefore likely that  $\text{Na}^+$  is released back into the soil subsequent to the death and decay of cyanobacteria.

In an alternative approach (Thomas, 1978; S. K. Apte and Joseph Thomas, unpublished results), saline 'Kharland' soils from coastal areas of Maharashtra were assessed for beneficial effects on inoculation with the salt tolerant cyanobacterium *A. torulosa*. These soils have much higher salinity levels (electrical conductivity:  $>15 \text{ mmhos.cm}^{-1}$ , or total soluble salts upto 10%) and are rich in sodium (upto 7% NaCl) (Joshi and Kadrekar, 1980). In laboratory experiments *A. torulosa* grew at moderate salinity levels (electrical conductivity:  $8.5 \text{ mmhos.cm}^{-1}$ ) which are likely to occur in inland soils adjoining coastal areas. After five weeks of growth there was considerable enrichment in the nitrogen status and subsequent removal of cyanobacterial mats from soils brought about 12–35% reduction in soil salinity (S. K. Apte and Joseph Thomas, unpublished results). Such treatment however removed most of the newly fixed nitrogen and carbon and the net gain in nitrogen and carbon content was only marginal ( $\approx 10\%$ ). Thus, if irreversible removal of salt has to be achieved the additional attraction of simultaneous enrichment of nitrogen and carbon status may have to be sacrificed. Moreover, although such a reclamation process appears to be comparable in its efficiency to some of the traditional methods, the ultimate removal of cyanobacterial mats physically appears impractical on a large scale.

The reported success of the approach used by Singh (1950, 1961) and recently by Kaushik and Venkataraman (1982) most probably lies in the apparent ability of cyanobacterial extracellular mucopolysaccharides to 'chelate' considerable amounts of various ions, especially  $\text{Na}^+$  (S. K. Apte and Joseph Thomas, unpublished results). Although such approach has no permanent solution to soil salinity it carries the dual benefits of temporarily immobilising the excess  $\text{Na}^+$  and of partly supplementing the nitrogen requirement of crops. A composite treatment involving both gypsum (to precipitate out  $\text{Na}^+$  salts which can be subsequently leached out by flooding) and cyanobacteria (to entrap and immobilise other salts) has recently been shown to be more effective in reclamation of sodic soils (Kaushik and Venkataraman, 1982). The remarkable ability of cyanobacteria, including fresh water forms, to withstand salt stress in the presence of combined nitrogen (S. K. Apte and Joseph Thomas, unpublished results) also promises to have good prospects in improvement of saline



soils. Possible reclamation of saline/alkali soils employing salt tolerant cyanobacteria along with nitrogenous fertilisers and also in combination with gypsum remains attractive and needs to be critically investigated.

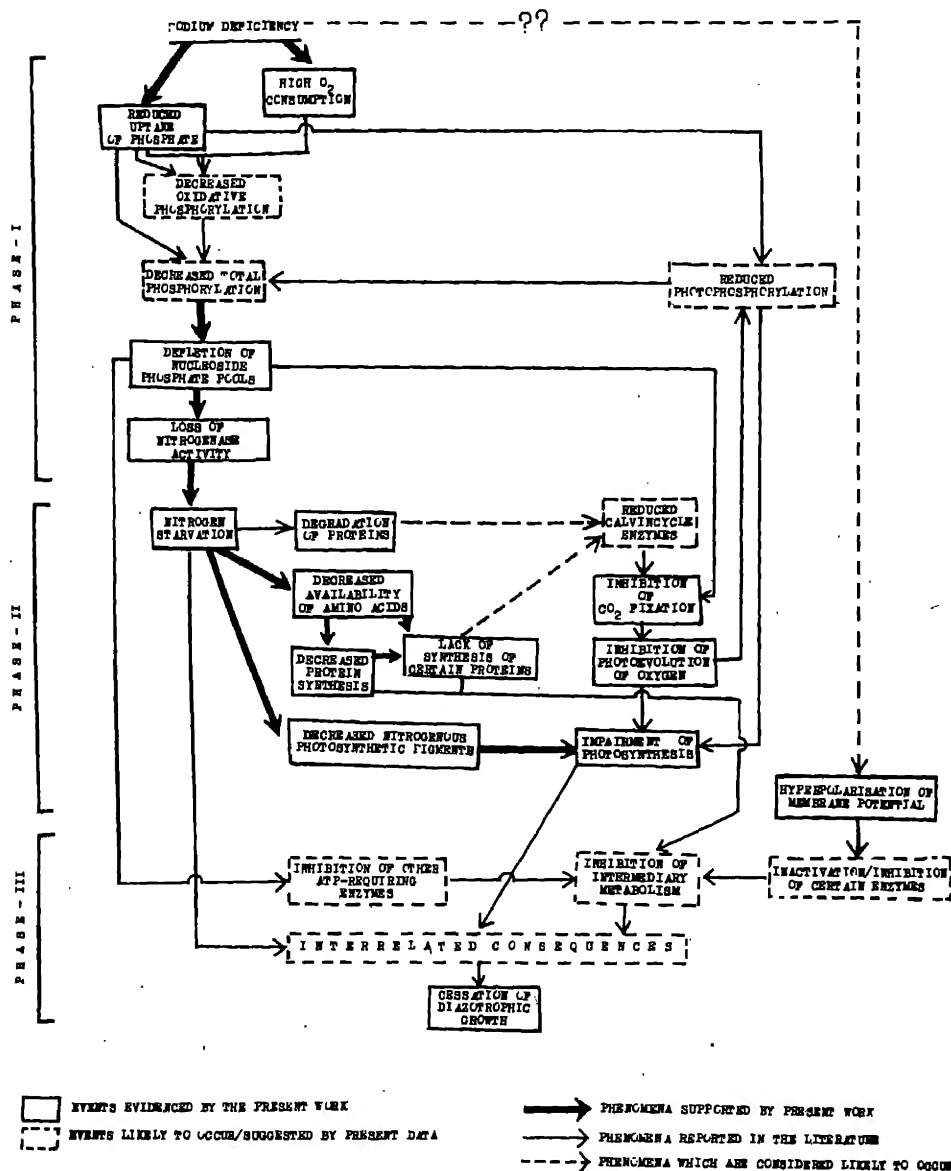


Figure 4. Probable sequence of events that result in retardation of growth of nitrogen-fixing *Anabaena torulosa* under sodium deficiency.

## Conclusions

There is a specific metabolic requirement of sodium ( $< 100 \mu\text{M}$ ) in cyanobacteria especially under  $\text{N}_2$ -fixing conditions. Although,  $\text{Na}^+$  does not influence several structural and functional features associated with diazotrophic growth like heterocyst differentiation, synthesis of nitrogenase proteins, transport of molybdenum and protection of nitrogenase from oxygen, vital functions like nitrogenase activity, photosynthesis, quality and quantity of proteins, membrane potential and energy status of  $\text{N}_2$ -fixing cells are affected by sodium deficiency. A primary effect of  $\text{Na}^+$  deficiency is inhibition of uptake and utilisation of phosphate leading to depletion of nucleotide phosphate pools. This results in inhibition of  $\text{N}_2$  fixation apparently due to limitation of ATP supply. The sequence of events that possibly results in inhibition of growth of  $\text{N}_2$ -fixing cyanobacteria under sodium deprivation is portrayed in the accompanying model (figure 4). A definite requirement for sodium also exists during nitrate-supported growth but its nature is not yet clear.

Extracellular mucopolysaccharides chelate significant amounts of sodium. Intracellular sodium exists as a free cation and is not incorporated into any biomolecule, especially proteins. It does not occur in storage products.  $\text{Na}^+$  influx in  $\text{N}_2$ -fixing *Anabaena* spp. is carrier-mediated and is regulated by the proton-motive force, particularly the membrane potential of cells. Low intracellular concentrations are maintained by active efflux. While the nature of this efflux is uncertain in  $\text{N}_2$ -fixing cyanobacteria, in *A. nidulans* it is mediated by  $\text{Na}^+/\text{H}^+$  antiporter and decreases the efficiency of oxidative phosphorylation.

Accumulation of  $\text{K}^+$ , exclusion of  $\text{Na}^+$  and maintenance of low intracellular  $\text{Na}^+$  levels, synthesis of carbohydrates, polyols, amino acids and quaternary amines for osmoregulation and other adaptations of metabolism are principal features associated with and contributing to the salt tolerance in cyanobacteria. The presence of combined nitrogen, which effectively curtails sodium accumulation and also supports the extra nitrogen demand for osmoregulation during salt stress, confers considerable salt tolerance on cyanobacteria. Exploiting the potential of cyanobacteria for reclamation of saline sodic soils needs more serious efforts than those made in the past.

Virtually no information exists on the genetics of cyanobacterial halotolerance. Genetic engineering of these photoautotrophic diazotrophs for enhanced halotolerance and subsequent agricultural exploitation is an attractive area of future research.

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## Molecular organization of great millet (*Sorghum vulgare*) DNA\*

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**Abstract.** Approximately 52% of the nuclear genome of great millet (*Sorghum vulgare*) consists of repetitive DNA which can be grouped into very fast, fast and slow components. The reiteration frequencies of the fast and slow reassociating components are 7000 and 92 respectively. Approximately 90% of the genome consists of repeated sequences interspersed amongst themselves and with single copy sequences. The interspersed repeat sequences are of three sizes viz. > 1.5 kilobase pairs, 0.5-1.0 kilobase pairs and 0.15-0.30 kilobase pairs while the size of the single copy sequences is 3.0 kilobase pairs. Hence the genome organization of great millet is essentially of a mixed type.

**Keywords.** Great millet genome; nuclear DNA content; DNA sequence organization.

### Introduction

Information about DNA sequence organization in eucaryotes is of fundamental importance as it provides a basis to understand several important parameters such as molecular events during evolution, origin of phenotypic variation and regulation of gene expression. In our laboratory, studies have been undertaken to analyse the genomes of a few Gramineae plant species with 1C nuclear DNA content less than 5 picograms (pg). The data on species with a DNA content of less than 5 pg like rice, finger millet and pearl millet (Gupta *et al.*, 1981; Gupta and Ranjekar, 1981, 1982) have shown that their DNA sequence organization not only differs amongst themselves but also from that of wheat (Flavell and Smith, 1976), rye (Smith and Flavell, 1977) and maize (Hake and Walbot, 1980). While the latter species, having a DNA content of more than 5 pg, exhibit predominantly a short period interspersion pattern, the former species show a varying interspersion pattern.

Great millet (*Sorghum vulgare*) is a commonly cultivated Gramineae plant species with a haploid nuclear DNA content of 4.6 pg (Joshi, 1982). Since no molecular data are available on this species, studies were undertaken to assess the effect of nuclear DNA content on its DNA sequence organization.

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Abbreviations used: pg, Picograms; PIPES, piperazine-N-N'-bis (2-ethanol sulphonic acid); Tris, Tris (hydroxy methyl amino methane); kbp, Kilobase pairs; *T<sub>m</sub>*, melting temperature; np, nucleotide pairs; HA, hydroxyapatite; RNase, ribonuclease.

### Materials and methods

All the chemicals used throughout the work were of Analytical or Guaranteed Reagent grade obtained from British Drug House, Bombay, Sarabhai chemicals and/or E. Merck. RNase, piperazine-N-N'-bis(2-ethanol sulphonic acid) (PIPES) and Tris (hydroxy methyl amino methane) (Tris) were obtained from Sigma Chemical Company, St. Louis, Missouri, USA.

#### DNA preparation

Eight day old seedlings were used for DNA extraction. Prior to homogenization, the tissue was frozen in liquid nitrogen and powdered in a Remi blender. The DNA isolation procedure was essentially that of Ranjekar *et al.* (1976) with a minor modification. Protein was extracted twice with freshly distilled phenol saturated with Tris-HCl (pH 7.5) followed by one chloroform/isoamyl alcohol (24:1) extraction. The optical properties of the DNA were as follows:

$$\frac{A_{230}}{A_{260}} = 0.45 \quad \frac{A_{280}}{A_{260}} = 0.55.$$

The absence of a 'foot' or shallow absorbance rise before the actual start of the DNA melting indicated the absence of single stranded DNA and RNA. The polysaccharide contamination in these preparations was negligible. Samples were routinely checked for their nativity on hydroxyapatite prior to reassociation and melting experiments.

#### Shearing and sizing of DNA

DNA fragments of an average length of 0.4 kilobase pairs (kbp) were obtained by sonication for 3 min using Biosonic III (Bronswell model, 250 W, 20 KHz) fitted with a half inch probe. Sonication with 1/4 inch probe for 1 min at 40 setting yielded DNA fragments of an average size of 1.5 kbp. DNA fragments in the range of 3.0–9.0 kbp were obtained using a Vir Tis 60 K homogenizer by varying the speed (30,000–58,000 rpm) and time (4–45 min). The DNA concentrations for sonication were kept constant at 400–450 µg/ml. The average size of the DNA fragments after shearing was determined by agarose slab gel electrophoresis using 1 % agarose with Hind III digest of bacteriophage λ DNA and Hae III digest of ϕ × 174 RF DNA, as molecular weight markers. The molecular weight of native unsonicated DNA was estimated to be nearly 20 kbp.

#### Reassociation techniques

The reassociation kinetics of great millet DNA of different fragment sizes (0.4 and 20 kbp) were studied essentially by the method of Britten *et al.* (1974). Kinetic fractions enriched in highly repetitive (very fast and fast) and intermediately repetitive (slow) sequences were also prepared (Hake and Walbot, 1980). The highly repetitive DNA included sequences which were bound to hydroxyapatite at Cot 0.5 M.s. DNA sequences which bind to hydroxyapatite at Cot 20 M.s. but not at Cot 0.5 M.s. were considered to be intermediately repetitive. These DNA fractions were analyzed for their

thermal stability and kinetic heterogeneity. To fractionate populations of repeated sequences by their extent of relatedness, reassociation of 0.4 kbp long great millet DNA was followed optically at different stringency criteria in the range of 55°–75°C.

### *Melting studies*

Thermal denaturation of total DNA and of repetitive DNA fractions was carried out as described earlier (Ranjekar *et al.*, 1976). In these studies, the data were used without any correction for solvent expansion. Single strand collapse was determined according to Graham *et al.* (1974) by melting unreassociated unique great millet DNA in 0.12 M sodium phosphate buffer (pH 6.8). The value obtained was in the range of 1.2–1.6%.

### *Sizing of repetitive DNA sequences*

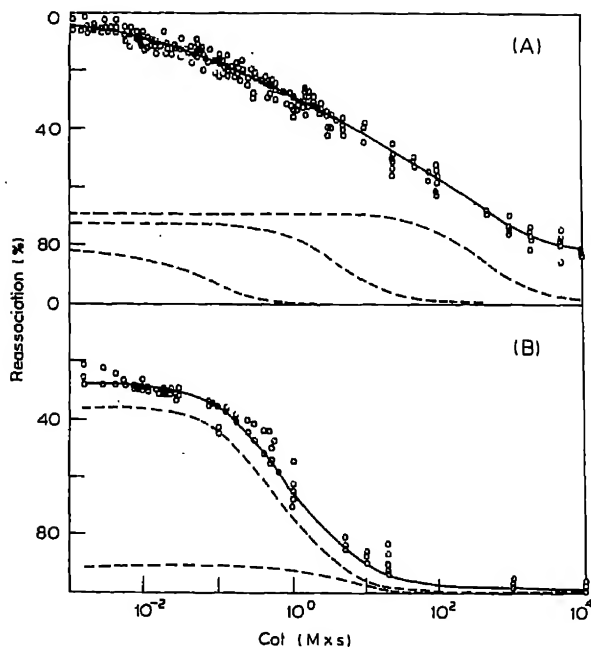
Native DNA of an approximate fragment length of 20 kbp was used for  $S_1$  nuclease digestion (Seshadri and Ranjekar, 1980). The size distribution of  $S_1$  resistant duplexes was determined by gel filtration on an Agarose A-50 (100–200 mesh size, Bio Rad Laboratories) column ( $92 \times 1.5$  cms) previously calibrated with calf thymus DNA of known duplex length. The column was developed in 0.12 M sodium phosphate buffer (pH 6.8).

## Results

### *Reassociation kinetics of short DNA fragments*

Reassociation kinetics of 0.4 kbp long DNA fragments of great millet DNA are given in figure 1A. A moderately stringent standard annealing criterion equivalent to 62°C and 0.18 M  $\text{Na}^+$  was employed for the construction of the reassociation curve. The data points were obtained using a combination of optical reassociation and hydroxyapatite chromatography. The curve was drawn through the data points by nonlinear least squares regression analysis, assuming second order kinetics and allowing all the parameters to free float. This analysis reveals that the curve can be modelled into three distinct second order components (root mean square = 0.027) differing in their reiteration frequency (table 1). About 6% of the genome reassociates very rapidly by  $\text{Cot } 1 \times 10^{-3}$  M.s. and could not be resolved with the techniques used in the present investigation. A fast component comprising 17.4% of the genome reassociates with an observed second order rate constant of  $1.12 \times 10^2$  and a reiteration frequency of 7000. The slow repetitive component comprising 27.4% of the genome has a second order rate constant of  $9.26 \times 10^{-1}$  and a reiteration frequency of 91. While approximately 31% of the genome contains sequences which reassociate with a rate constant of  $8.97 \times 10^{-3}$  indicating the presence of mainly single copy sequences, the remaining 18% of the genome fails to reassociate at a  $\text{Cot}$  value of  $1 \times 10^4$  M.s. This is attributed to DNA degradation resulting from long incubations and failure of some sequences to form stable duplexes at the above experimental conditions.





**Figure 1.** Reassociation kinetics of (A) 0.4 kbp long and (B) unsonicated 20 kbp long DNAs. The solid lines through the data points represent the least squares fit allowing all parameters to free float (root mean square, 0.027 for A and 0.061 for B). The lower dashed curves represent the reassociation kinetics of pure components.

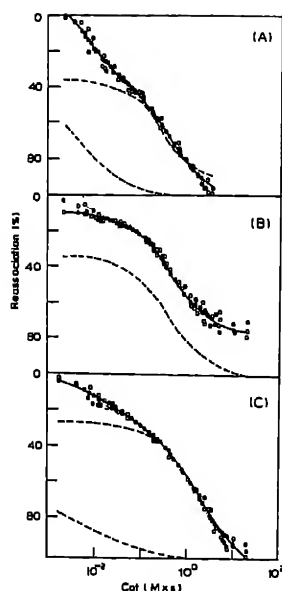
### *Reassociation of fractions enriched with repetitive DNA sequences*

For a direct demonstration of the existence of discrete families of nucleotide sequences with respect to repetition frequency, kinetics of reassociation of isolated fractions enriched in highly and intermediately repetitive DNA sequences were studied. Computer analysis of the mini-Cot curve of the highly repetitive DNA demonstrates the presence of two components (figure 2A and table 2). The faster of the two components, almost 40% of the total, has a very low Cot 1/2 pure value of  $4.8 \times 10^{-3}$  M.s. It contains sequences with a high copy number of 74,000 and a kinetic complexity of  $1.5 \times 10^3$  nucleotide pairs. The slower component of the fraction is typical of the intermediately repetitive class with a Cot 1/2 pure value of  $2.31 \times 10^{-1}$  M.s. and a kinetic complexity of  $1.93 \times 10^5$  nucleotide pairs. The intermediately repetitive class (figure 2B) could be modelled with only a single component with a Cot 1/2 pure value of  $2.69 \times 10^{-1}$  M.s. and a kinetic complexity of  $2.24 \times 10^5$  nucleotide pairs. The kinetics of total repetitive DNA (figure 2C) show the presence of two components with reiteration frequencies of 2620 and 256 and kinetic complexities of  $2.93 \times 10^3$  and  $8.58 \times 10^5$  nucleotide pairs respectively. From the analysis of the mini-Cot curves, it is evident that each fraction is contaminated to some extent with the other. This is partly due to the fact that highly repetitive and intermediately repetitive DNA sequences are interspersed.

Table 1. Kinetic analysis of 0.4 kbp and 20 kbp long great millet DNA.

Fraction <sup>a</sup> of the total DNA	Cot 1/2 <sup>a</sup> values observed (M.s.)	Cot 1/2 <sup>b</sup> values pure M.s.	K pure <sup>c</sup>	Kinetic complexity in base pairs <sup>d</sup>	Frequency of repetition per IC genome <sup>e</sup>
<b>0.4 kbp long DNA</b>					
Very fast	—	—	—	—	—
Cot < 1 × 10 <sup>-3</sup> M.s.	—	—	—	—	—
Fast	—	—	—	—	—
Cot 1 × 10 <sup>-3</sup> to 5 × 10 <sup>-1</sup> M.s.	5.17 × 10 <sup>-2</sup>	8.97 × 10 <sup>-3</sup>	1.12 × 10 <sup>2</sup>	6.94 × 10 <sup>3</sup>	7.62 × 10 <sup>3</sup>
Intermediate	—	—	—	—	—
Cot 5 × 10 <sup>-1</sup> to 2.0 × 10 <sup>1</sup> M.s.	3.92 × 10 <sup>0</sup>	1.08 × 10 <sup>0</sup>	9.26 × 10 <sup>-1</sup>	3.40 × 10 <sup>6</sup>	9.17 × 10 <sup>1</sup>
Unique	—	—	—	—	—
Cot 2.0 × 10 <sup>1</sup> to 1 × 10 <sup>4</sup> M.s.	3.59 × 10 <sup>2</sup>	1.11 × 10 <sup>2</sup>	8.97 × 10 <sup>-3</sup>	3.05 × 10 <sup>8</sup>	1.00 × 10 <sup>0</sup>
Unreassociated	—	—	—	—	—
Cot > 1 × 10 <sup>4</sup> M.s.	—	—	—	—	—
<b>20 kbp long DNA</b>					
Very fast Cot < 1.6 × 10 <sup>-3</sup> M.s.	—	—	—	—	—
Fast Cot 1.6 × 10 <sup>-3</sup> to 2.0 × 10 <sup>1</sup> M.s.	—	—	—	—	—
Intermediate	—	—	—	—	—
Cot 2.0 × 10 <sup>1</sup> to 1.0 × 10 <sup>4</sup> M.s.	7.14 × 10 <sup>-1</sup>	4.57 × 10 <sup>-1</sup>	2.19 × 10 <sup>0</sup>	3.88 × 10 <sup>5</sup>	5.48 × 10 <sup>2</sup>
Unreassociated Cot > 1.0 × 10 <sup>4</sup> M.s.	3.91 × 10 <sup>2</sup>	3.27 × 10 <sup>1</sup>	3.06 × 10 <sup>-2</sup>	2.78 × 10 <sup>7</sup>	1.00 × 10 <sup>0</sup>

<sup>a</sup> Values obtained from figure 1.<sup>b</sup> Cot 1/2 values observed × fraction of genome.<sup>c</sup> K pure = (Cot 1/2 pure)<sup>-1</sup>.<sup>d</sup> Values obtained by comparing the Cot 1/2 of 5.3 M.s. under our experimental conditions and genomes size 4.5 × 10<sup>6</sup> base pairs of *Escherichia coli*.<sup>e</sup> Cot 1/2 of unique DNA divided by Cot 1/2 values of the given fraction.



**Figure 2.** Reassociation kinetics of (A) highly repetitive (Cot < 0.5 M.s.) (B) intermediately repetitive (Cot 0.5–20 M.s.) and (C) total repetitive (Cot 20 M.s.) fractions isolated from 0.4 kbp long great millet DNA as described earlier in Materials and methods. The solid lines through the data points represent the best least squares solutions for the three fractions.

### *Melting properties of total DNA and repetitive DNA fragments*

Native great millet DNA (20 kbp long) melts in 0.12 M sodium phosphate buffer, pH 6.8 with a melting temperature ( $T_m$ ) of 87.7°C and an average hyperchromicity of 25%. The optical dispersion, defined by Mahler and Dutton (1964) is estimated to be 7.5°C and the G+C content as estimated from its  $T_m$  is 44.9%. Sonicated DNA (0.4 kbp) has a  $T_m$  of 86.9°C. The Cot  $5 \times 10^{-1}$  fraction isolated on hydroxyapatite, which mainly includes foldback sequences and highly repetitive DNA, has a thermal stability ( $T_m$  80.6°C) lower than that of native DNA and shows a sequence divergence of 6.84%. The intermediately repetitive DNA (Cot 0.5–2.0) has a  $T_m$  of 77.25°C and a sequence divergence of 10% (table 3).

### *Kinetic analysis of 0.4 kbp long DNA at different stringency criteria of temperature*

The optical reassociation of 0.4 kbp long great millet DNA was studied till a Cot value of  $3.0 \times 10^0$  M.s. at 55°, 68° and 75°C apart from the standard criterion of 62°C. By Cot  $3.0 \times 10^0$  M.s., all the highly repetitive and a part of the intermediately repetitive DNA sequences reassociate. The reassociation behaviour of these DNA sequences under different stringency conditions was compared with that at the standard criterion of 62°C. It can be seen from figure 3 that the reassociation curves obtained at 55°C and 62°C could be resolved into two components while those at 68°C and 75°C showed only a single component. The rate constant obtained for the fast reassociating component at

Table 2. Kinetic analysis of mini Cot curves.

	Fraction <sup>a</sup>	Cot 1/2 <sup>a</sup> values observed (M.s.)	Cot 1/2 <sup>b</sup> values pure (M.s.)	K pure <sup>c</sup>	Frequency <sup>d</sup> of repetition per 1C genome	Kinetic <sup>e</sup> complexity in base pairs
<i>Cot 0.5 fraction</i>						
Very fast	0.37	$4.84 \times 10^{-3}$	$1.77 \times 10^{-3}$	$5.65 \times 10^2$	74174	$1.48 \times 10^3$
Cot $2.3 \times 10^{-3}$ to $4.0 \times 10^{-2}$ M.s.						
Fast	0.64	$3.61 \times 10^{-1}$	$2.31 \times 10^{-1}$	$4.33 \times 10^0$	995	$1.93 \times 10^3$
Cot $4.0 \times 10^{-2}$ to $3.0 \times 10^0$ M.s.						
<i>Cot 0.5-Cot 20 fraction</i>						
Fast	0.66	$4.08 \times 10^{-1}$	$2.69 \times 10^{-1}$	$3.72 \times 10^0$	880	$2.24 \times 10^3$
Cot $2.0 \times 10^{-3}$ to $2.0 \times 10$ M.s.						
<i>Cot 20 fraction</i>						
Very fast	0.26	$1.37 \times 10^{-2}$	$3.51 \times 10^{-3}$	$2.85 \times 10^2$	2620	$2.93 \times 10^3$
Cot $1.6 \times 10^{-3}$ to $1.13 \times 10^{-1}$ M.s.						
Fast	0.73	$1.40 \times 10^0$	$1.03 \times 10^0$	$9.71 \times 10^{-1}$	256	$8.58 \times 10^3$
Cot $1.13 \times 10^{-1}$ to $2.00 \times 10^1$ M.s.						

<sup>a</sup> Values obtained from figure 3. <sup>b</sup> Cot 1/2 value observed  $\times$  fraction of genome.<sup>c</sup> K Pure = (Cot 1/2 pure)<sup>-1</sup>. <sup>d</sup> Cot 1/2 of unique DNA obtained from figure 2A divided by Cot 1/2 value of the given fraction.<sup>e</sup> Values obtained by comparing to Cot 1/2 of 5.3 M.s. under our experimental conditions and genome size  $4.5 \times 10^6$  base pairs of *E. coli*.

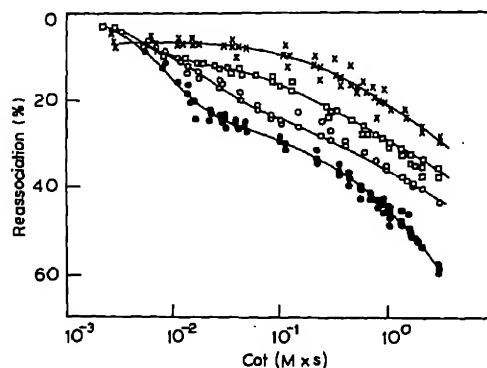
**Table 3.** Melting analysis of total DNA and repetitive DNA fractions.

DNA	$T_m$ (°C)	Hyperchromicity <sup>a</sup> (%)	Base mismatch <sup>b</sup> (%)
Unsonicated 20 kbp long DNA	87.80 ± 0.21	24.88 ± 2.5	—
Native 0.4 kbp long DNA	86.90 ± 0.90	23.80 ± 1.0	—
Cot 0.5 DNA	80.06 ± 0.84	17.30 ± 0.8	6.84
Cot 0.5–20 DNA	77.25 ± 0.45	20.57 ± 1.2	9.65
Cot 20 DNA	79.55 ± 0.25	18.25 ± 0.7	7.35

<sup>a</sup> Hyperchromicity (H) was calculated using the formula:

$$H = \frac{A_{260}(98^\circ\text{C}) - A_{260}(60^\circ\text{C})}{A_{260}(98^\circ\text{C})}$$

<sup>b</sup> Per cent base mismatching is calculated as: lowering in  $T_m$  by 1°C corresponds to 1% base mismatch (Ullman and McCarthy, 1973).



**Figure 3.** Optical reassociation kinetics of 0.4 kbp long great millet DNA followed upto Cot 3.0 M.s. at different conditions of stringency. (X), 75°C; (□), 68°C; (O), 62°C; (●) 55°C. The solid lines through the data points represent the best least squares solutions.

62°C is compared with the rates of the single component obtained at 68° and 75°C and the rate of the fast component obtained at 55°C, as the second component did not reassociate completely at a Cot value of  $3.0 \times 10^0$  M.s. It can be seen from figure 3 that when DNA reassociation is studied at 75°C, the extent of reassociation at Cot 3 reduces from 60% to 30%, indicating that at this temperature some of the repeated sequences no longer behave as repetitive. After the reassociation rate is corrected for the effect of temperature and the decreased extent of reaction (Bonner *et al.*, 1973; Braun *et al.*, 1978), it can be seen from table 4 that the observed rate of reaction shows a 35 fold decrease (from 60.38 to 1.73) as the reassociation temperature increases from 62° to 75°C. This is because at a higher temperature, only well matched duplexes reanneal ( $T_m$  83.5°C) indicating an increase in their specificity. At a lower reassociation temperature, the rate decreases (9.44 M.s. at 55°C) 6 fold but duplexes formed show

mismatch ( $T_m$  76.5°C) indicating decreased specificity. Melting analysis of the duplexes formed under different stringency criteria of temperature (table 4) shows that the duplexes formed at 75°C are more stable than those at 68°, 62° or 55°C.

#### *Reassociation kinetics of long DNA fragments*

The reassociation kinetics of 20 kbp great millet DNA is shown in figure 1B and the results are summarized in table 1. The repetitive DNA could be resolved only as a single component at this fragment length indicating that the highly repetitive and intermediately repetitive DNA components are linked together. Extensive interspersions of these components indicates the fact that at a  $Cot$  value of  $5 \times 10^{-1}$  M.s. the binding of duplexes to hydroxyapatite increases from 25 to 55%. At the repetitive  $Cot$  value of 20 M.s., the per cent reassociation increases from 52 to 92%. This suggests that the repetitive components are also extensively interspersed with single copy DNA sequences in the great millet genome. There is also an increase in the DNA binding to hydroxyapatite from 6 to 30% at a low  $Cot$  value of  $1 \times 10^{-3}$  M.s. This is attributed to the interspersions of very highly repetitive and/or foldback sequences along with other repetitive and single copy sequences.

#### *Hyperchromicity of reassociated duplexes*

The reassociated DNA duplexes from 0.4 kbp, 1.5 kbp and 20 kbp long DNA fragments were isolated at  $Cot$  20 M.s. and melted to determine their thermal stability and hyperchromicity. From table 5, it can be seen that the  $T_m$  of the reassociated duplexes (85°–87°C) from 1.5 and 20 kbp DNA is very close to that of the native unsheread DNA, whereas the  $T_m$  of those isolated from 0.4 kbp DNA is much lower (79.5°C). Long repetitive duplexes thus have a greater thermal stability than the shorter ones. The hyperchromicity of the 0.4 kbp duplexes is 19.5% in contrast to 14% for 20 kbp fragments. This is mainly due to the increasing presence of single stranded regions in the reassociated fragments. Using the hyperchromicity values, it is estimated that 84% of the 0.4 kbp long DNA and 58% of the 20 kbp long fragment is base paired. This is expected if there is extensive interspersions of single copy and repetitive DNA sequences.

#### *Size distribution of repetitive and single copy DNA duplexes*

An independent approach to determine the presence of single strand regions in the reassociated duplexes is to subject the duplexes from 20 kbp DNA to  $S_1$  nuclease digestion and study the melting properties of the  $S_1$  resistant fraction. Approximately 55% of the 20 kbp DNA which binds to hydroxyapatite at  $Cot$  20 M.s. is  $S_1$  resistant and hence in the duplex form. The hyperchromicity of the  $S_1$  resistant DNA is 23.6% as against 14% of the duplexes not treated with  $S_1$  nuclease.

To estimate the size distribution of repeated DNA sequences, the  $S_1$  resistant duplexes were fractionated on Agarose A-50 Biogel column. From figure 4B, it is clear that the repetitive duplexes have a heterogeneous size distribution and can be grouped into three classes. The proportion of the excluded DNA fraction ( $> 1.5$  kbp) ranges from 43 to 48%. The second fraction which accounts for 32–38% consists of DNA

Table 4. Analysis of 0.4 kbp great millet DNA by optical reassociation and melting at different criteria of temperature stringency.

Incubation temperature and Cot range	Fraction <sup>a</sup>	Cot 1/2 <sup>b</sup> observed (M.s.)	K <sup>c</sup> observed	T <sub>m</sub> (°C)	T <sub>p</sub> <sup>d</sup> (°C)	T <sub>i</sub> -T <sub>p</sub> <sup>e</sup>	Rate <sup>d</sup> Relative to optimum rate	Adjusted <sup>f</sup> K	Kinetic <sup>g</sup> complexity in base pairs	Frequency of <sup>h</sup> repetition per 1C genome
55°C (RMS = 0.014)										
1.6 × 10 <sup>-2</sup> - 4.27 × 10 <sup>-1</sup>	0.15	9.63 × 10 <sup>-2</sup>	10.38	76.5	81.7	-26.70	1.10	9.44	1.36 × 10 <sup>4</sup>	3095
4.27 × 10 <sup>-1</sup> - 3.00 × 10 <sup>0</sup>	0.64	5.08 × 10 <sup>0</sup>	0.20					0.18	3.02 × 10 <sup>6</sup>	20
62°C (RMS = 0.026)										
3.2 × 10 <sup>-2</sup> - 5.00 × 10 <sup>-1</sup>	0.28	1.69 × 10 <sup>-2</sup>	59.17	79.4	83.15	-21.15	0.98	60.38	4.04 × 10 <sup>3</sup>	6731
5.0 × 10 <sup>-1</sup> - 3.00 × 10 <sup>0</sup>	0.31	2.69 × 10 <sup>0</sup>	0.37					0.38	6.93 × 10 <sup>5</sup>	42
68°C (RMS = 0.020)										
2.2 × 10 <sup>-3</sup> - 3.00 × 10 <sup>0</sup>	0.29	2.54 × 10 <sup>-1</sup>	3.94	82.0	84.45	-16.45	0.75	5.25	4.67 × 10 <sup>4</sup>	585
75°C (RMS = 0.026)										
2.6 × 10 <sup>-3</sup> - 3.00 × 10 <sup>0</sup>	0.30	9.04 × 10 <sup>-1</sup>	1.11	83.5	85.20	-10.20	0.64	1.73	1.47 × 10 <sup>5</sup>	193

<sup>a, b</sup> Values obtained from the optical reassociation curves in figure 3.<sup>c</sup> K = 1/Cot 1/2 observed.<sup>d</sup> T<sub>m</sub> of duplexes formed after reassociation at different criteria of temperature stringency.<sup>e</sup> T<sub>p</sub> = (T<sub>m</sub> + T<sub>i</sub>)/2. T<sub>i</sub> is the melting temperature of well matched DNA, 86.9°C for 0.4 kbp long great millet DNA.<sup>f</sup> T<sub>i</sub> = incubation temperature.<sup>g</sup> Taken from figure 8 of Bonner et al. (1973).<sup>h</sup> Calculated from (observed rate)/(rate relative to optimum rate).<sup>i</sup> Values obtained from the standard relationship for *E. coli* DNA where Cot 1/2 is 5.3 (under our experimental conditions) and the number of nucleotide pairs is 4.5 × 10<sup>6</sup>.<sup>j</sup> Cot 1/2 value of unique DNA as described in table 1 (0.4 kbp long) divided by Cot 1/2 value of given fraction.

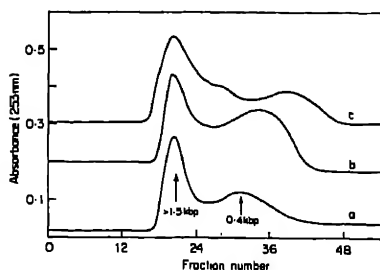


Figure 4. Agarose A-50 column chromatographic profiles of (a) calf thymus DNA of known duplex lengths (b)  $S_1$  nuclease resistant Cot 20 great millet DNA and (c)  $S_1$  nuclease resistant Cot 0.1 great millet DNA.

sequences of 0.15–0.30 kbp. The third class of repetitive duplexes has a size distribution in the range of 0.5 to 1.0 kbp and accounts for about 14–25% of the total duplexes.

Experiments were also carried out where the DNAs of high molecular weight ( $> 20$  kbp) were reassociated to Cot 0.1 M.s. and then treated with  $S_1$  nuclease and the  $S_1$  resistant duplexes fractionated on Agarose A-50 to determine the distribution of long and short repeats in the highly repetitive fast reassociating fraction (figure 4, profile C). Three distinct sizes of repeat classes are observed in the elution profile. The proportion of the short repeats is about 28–34%, that of the intermediate repeats is about 28–34% and that of the long repeats in 35–41% (table 6).

The length of interspersed single copy sequences can be estimated from a curve relating the fraction of DNA fragments binding to hydroxyapatite as a function of DNA fragment length. In figure 5, two slopes are observed and the change in slope occurs at a fragment length of 2.5–3.0 kbp which is the estimated length of the single copy DNA. A more gradual increase in slope after 3.0 kbp indicates the presence of a spectrum of single copy sequences with lengths greater than 3.0 kbp. Extrapolation of the line with the greater slope to the ordinate indicates the proportion of repetitive DNA in the great millet genome to be 54%.

## Discussion

The present studies have yielded information about the gross organization of the nuclear DNA of great millet. The repetitive DNA content as determined by the reassociation kinetics of 0.4 kbp long DNA is 52%. This value compares well with those obtained using other experimental approaches cited in the present investigation (table 7). Mini Cot curves have enabled us to detect the presence of a very fast reassociating DNA fraction containing DNA sequences with high reiteration frequency ( $7.4 \times 10^4$ ) and an average complexity of  $2.3 \times 10^3$  nucleotide pairs. Melting analysis of enriched fractions indicates that the highly repetitive sequences are more stable than the intermediately repetitive DNA. Studies on reassociation at different stringency conditions have demonstrated that stable families reassociating at a higher temperature have lower copy number and reassociation rate than those reassociating at a lower temperature. It, therefore, appears that repeat families in the great millet



Table 5. Melting analysis of Cot 20 DNA isolated from DNAs of different fragment lengths.

DNA fragment size (nucleotide pairs)	400	1500	5600	8200	20000	Native DNA (0.4 kbp)
Fraction bound to HA at Cot 20 <sup>a</sup>	0.5	0.60	0.815	0.865	0.935	—
Hyperchromicity <sup>b</sup>	19.5 ± 2.63	20.1 ± 0.15	18.6 ± 1.12	17.8 ± 1.90	14.0 ± 4.6	23.0 ± 0.8
<i>T<sub>m</sub></i> °C	79.5 ± 0.19	86.2 ± 0.15	86.4 ± 1.12	86.9 ± 0.2	87.0 ± 0.3	—
Duplex content (D) <sup>c</sup>	0.839	0.867	0.798	0.761	0.586	—
Average duplex length <sup>d</sup>	335.6	1300.5	4468.8	6240.2	11720.0	—
Duplex content from S <sub>1</sub> nuclease <sup>e</sup>	—	—	—	—	0.550	—

<sup>a</sup> Obtained from figure 5.<sup>b</sup> Hyperchromicity (H) was calculated using the formula:

$$H = \frac{A_{260}(98^{\circ}\text{C}) - A_{260}(60^{\circ}\text{C})}{A_{260}(98^{\circ}\text{C})} \quad (\text{Zimmerman and Goldberg, 1977; Wimpee and Rawson, 1977}).$$

<sup>c</sup> The average duplex content (D) of bound fragment was estimated using the formula

$$D = \frac{H - \text{single strand collapse}}{H (\text{native sonicated DNA}) - \text{Single strand collapse}}.$$

<sup>d</sup> The average length of duplex region is the product of the duplex content (D) and the fragment length of DNA.<sup>e</sup> Duplex content of S<sub>1</sub> nuclease treated repetitive DNA fragment was obtained as:

$$\frac{\mu\text{g of S}_1 \text{ nuclease resistant repetitive duplexes bound to hydroxyapatite column}}{\mu\text{g of S}_1 \text{ nuclease treated total DNA loaded on hydroxyapatite column}}.$$

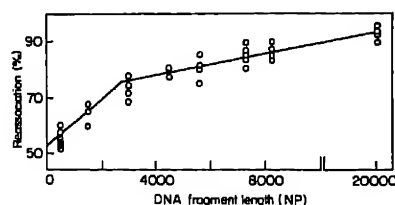


Figure 5. The percentage binding of great millet DNA to hydroxyapatite at Cot 20 M.s. as a function of DNA fragment length.

Table 6. Size distribution of  $S_1$  resistant fractions.

Cot value M.s.	Fraction <sup>a</sup> of long repeats (%)	Fraction <sup>b</sup> of repeats of intermediate length (%)	Fraction <sup>c</sup> of short repeats (%)
0.1	35-41	28-34	28-34
20.0	43-48	32-38	14-25

<sup>a, b, c</sup> Obtained from figure 4.

Table 7. Duplex content of repetitive DNA fractions of great millet from independent experimental approaches.

Method	Duplex content
Reassociation curve of 0.4 kbp long DNA (figure 1A)	0.520
Hyperchromicity measurements (table 5)	0.586
$S_1$ nuclease resistance measurements (table 5)	0.550
Reassociation (%) vs fragment length (figure 5)	0.540

genome are essentially heterogeneous (Bendich and Anderson, 1977; Bendich, 1979; Preisler and Thompson, 1981). The increased complexity and decreased frequency of repetition, when reassociation is carried out under rigorous criteria, indicate that the family members of the highly repetitive class in the great millet genome are only broadly related. The minimum estimate of repeated sequence copy number is 193. They may represent a cryptic class of repeated sequences undetected in the reassociation kinetics of total 0.4 kbp long great millet DNA.

The reassociation kinetics data of long DNA fragments, hyperchromicity measurements of reassociated DNAs of different fragment lengths and  $S_1$  nuclease experiments have provided a clear evidence about the occurrence of extensive interspersion of repeated and single copy DNA sequences in the great millet genome. Table 8 summarizes the available DNA sequence organization data in the family Gramineae.

Table 8. Genome organization in a few Gramineae plant species.

Species	DNA content 1C (pg)	Repetitive DNA content (%)	Length of interspersed repetitive DNA np	Length of interspersed single copy DNA np	Pattern of interspersion	Reference
Wheat <i>Triticum aestivum</i>	18.10	75.0	350-650 (32%) 400-800 (60%) Long repeats (10%)	1000 (88%) 1000 (12%)	Predominantly short	Flavell and Smith 1976
Rye <i>Secale cereale</i>	9.00	70.0	550-680 (Major) 2000-3000 (Minor)	1500	Predominantly short	Smith and Flavell, 1977
Maize <i>Zea mays</i>	7.70	58.2	300-1000	2100	Short	Hake and Walbot, 1980
Finger millet <i>Eleusine coracana</i>	1.60	49.0	150-200 (57-64%) 500-1000 (18-20%) 4000-4200 (18-23%)	1900	Mixed	Gupta and Ranjekar, 1981
Pearl millet <i>Pennisetum americanum</i>	2.50	54.0	4300-4500	1900	Long	Gupta and Ranjekar, 1982
Rice <i>Oryza sativa</i>	0.60	52.0	50-100 (60-65%) 6000-6400 (35-40%)		Absent	Gupta et al., 1981

From this table, it can be seen that the lengths of the interspersed repeated DNA sequences are diverse, varying in the range of 50–6400 nucleotide pairs (np) while that of the interspersed single copy DNA varies in a narrow range of 1000–2000 np. Great millet has essentially a mixed type of DNA sequence organization pattern. This is in keeping with the trend that plants with 1C nuclear DNA content of less than 5 pg have diverse patterns of DNA sequence organization while those with 1C nuclear DNA content greater than 5 pg have predominantly a short period interspersion pattern. The above observations lead to the question whether nuclear DNA content plays a role in determining the sequence organization pattern in plants and if so what is its significance. Attempts in this direction are in progress.

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## Increased circulatory half-life of liposomes after conjunction with dextran

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**Abstract.** Dextran was covalently coupled to neutral unilamellar liposomes. Dextran conjugated liposomes were cleared from the circulation at a much slower rate than unconjugated liposomes. The uptake of dextran conjugated liposomes by liver and spleen was also decreased. The amount of dextran on the surface of liposomes was found to be a determining factor for their stability in circulation. Dextran conjugated liposomes therefore may be a more effective way of controlled drug release.

**Keywords.** Unilamellar liposomes; dextran conjugate; drug delivery.

### Introduction

Liposomes are becoming increasingly important as carriers of biologically important molecules in living system (Tyrell *et al.*, 1976; Papahadjopoulos, 1978; Gregoriadis, 1980; Roerdink *et al.*, 1981). Introduction of a specific sugar onto the liposomal surface either by specific glycolipids or by coupling *p*-aminophenyl glycosides helps in directing the liposome towards different cell types of liver (Ghosh and Bachhawat, 1980; Ghosh *et al.*, 1981a, 1982). Glycoside-bearing liposomes have been used to deliver therapeutic substances in galactosamine induced hepatitis (Ghosh *et al.*, 1981b). Although drugs in general usually must quantitatively arrive at their target, drug-bearing liposomes, however, which have extremely long half-lives in the circulation could enhance the treatment of some of the diseases involving lack of serum factors or can serve as intravascular sustained release of antitumor drugs and other agents. Dextran has long been used as drug carrier to confer greater chemical and biological stability to dextran-drug complex (Molteni, 1979). Dextran remains in the blood stream for periods of time proportional to its molecular weight. In this paper we have conjugated dextran with

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Abbreviations used: Chol, Cholesterol; EL, egg lecithin; PE, phosphatidylethanolamine; ConA, concanavalin A

liposomes and have shown that such dextran conjugated liposomes can serve the dual purpose of extending the duration of drugs in the circulation considerably and of protecting the drug from the hostile environment.

## Materials and methods

### Chemicals

Egg lecithin, cholesterol and phosphatidylethanolamine were obtained from CSIR Centre for Biochemicals, New Delhi. Sepharose-6B, bovine  $\gamma$ -globulin and dextran of mol. wt. 70,000 were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Carrier-free Na [ $^{125}$ I] was obtained from BARC, Bombay. Concanavalin A (Con A) was prepared according to the method of Surolia *et al.* (1973). All other chemicals used were of analytical grade.

### Radioiodination of $\gamma$ -globulin

Radioiodination of  $\gamma$ -globulin was done by the Chloramine-T method of Hunter (1978) using carrier-free Na [ $^{125}$ I]. The iodinated protein was separated from free iodide by gel filtration on Sephadex G-25. The specific activity of iodinated protein was  $2.2 \times 10^6$  cpm/ $\mu$ g.

### Preparation of liposome

[ $^{125}$ I]-Labelled  $\gamma$ -globulin entrapped unilamellar liposomes were prepared with egg lecithin (EL), cholesterol (Chol) and phosphatidylethanolamine (PE) as described earlier (Surolia *et al.*, 1975).

### Covalent coupling of dextran to PE-liposome

Covalent coupling of dextran to the available amino groups on PE-liposome was done by a modification of the method described for dextran-protein conjugates (Marshall and Rabinowitz, 1976). Cyanogen bromide (30–35 mg) was added to a stirred solution of dextran (250 mg) in 5 ml of distilled water, adjusted to pH 10.7 with 1 M NaOH, followed by a second addition of cyanogen bromide (30–35 mg), 30 min later. The pH was maintained at 10.7 by 1 M NaOH throughout the activation. Thirty minutes after the second addition, cyanogen bromide (20–25 mg) was again added and allowed to react for another 20 min at pH 10.7. The reaction mixture was then dialysed at 4°C for 1 h against 0.15 M NaCl, pH 9.0, adjusted with Na<sub>2</sub>CO<sub>3</sub>. The dialysate was added to 3–4 ml of liposome preparation containing entrapped [ $^{125}$ I]- $\gamma$ -globulin and the mixture was stirred overnight at 4°C. The excess unreacted dextran was blocked by the addition of 0.2 ml of dilute ethanolamine (1:25). After stirring for another 2 h, the conjugate was purified by gel filtration on Sepharose-6B.

### Animal experiments

Male Swiss albino mice (IICB strain) weighing approx. 25–30 g were used throughout the experiment. Each pentobarbital anaesthetized mouse received a single intravenous

injection of 0.25 ml liposome suspension (1–1.5 mg lipids) containing  $3-6 \times 10^4$  cpm entrapped [ $^{125}\text{I}$ ]- $\gamma$ -globulin. After time intervals of 2.5, 5, 15, 30, 60, and 120 min, samples of blood were withdrawn from the femoral artery of groups of three mice. Subsequently they were sacrificed and their livers, kidneys, spleens and lungs were removed. Each tissue was washed with saline and blotted with filter paper. The whole liver was digested with 5 ml of 30% KOH solution and 2 ml of the liver-digest was taken for radio-active counting. Other tissues were digested in 1 ml of 30% KOH solution and counted. Radioactivity was measured in a Prias Scintillation gamma counter.

## Results and discussions

We have investigated the effect of dextran conjugation to small unilamellar liposomes on the rate of clearance of their entrapped solute contents from the blood and also the uptake by different tissues of the injected animals. [ $^{125}\text{I}$ ]- $\gamma$ -Globulin was taken as the entrapped marker.

### *Dextran-liposome conjugate*

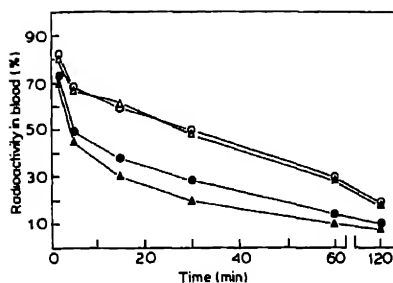
The dextran liposome conjugate was freed from any unreacted dextran by gel filtration on Sepharose-6B and subsequently characterized by its agglutination by ConA. Different amounts of PE (10, 18 and 30 mol %) were taken for liposome preparation and were conjugated with the same amount of dextran (250 mg). When all types of conjugates were separately agglutinated by ConA followed by centrifugation, the radioactivity present in the supernatant fractions was negligible showing thereby that the conjugate preparation does not contain any unreacted liposome. This can be explained due to the use of excess dextran over the available amino groups on the PE liposome. However, as expected the conjugate preparations differ significantly in their dextran content and this was monitored by the per cent of total amino groups available even after the coupling, as estimated by the titrations of the liposomal PE amino groups with trinitrobenzenesulphonic acid in the presence of 0.1 % Triton X-100 (Torchillin *et al.*, 1978). In addition, when the purified liposome-dextran conjugate in 6 M urea was rechromatographed on Sepharose-6B to remove contaminated noncovalent material, if any, no peak other than the conjugate was obtained. Therefore, the presence of any non-specifically adsorbed dextran on liposomal surface is quite unlikely.

### *Blood clearance and tissue uptake studies*

Figure 1 shows the rates of clearance of entrapped protein from the circulation of mice. Dextran conjugated PE-liposomes were found to be much more efficient in retaining the radioactivity in blood stream compared to control liposomes (PE-liposomes). About 80% of the control liposomes are removed from the circulation after 30 min of injection, whereas only 50% of the injected dextran-liposomes are cleared during this period. The difference in the retention in circulation of dextran-liposome is found to be 2-fold higher even after 2 h of intravenous injection (18% and 8% respectively).

In another control experiment, mice were injected with 0.25 ml of a mixture of





**Figure 1.** Clearance of different types of liposomes from the circulation of mice. Mice were injected with liposomes containing [ $^{125}$ I]- $\gamma$ -globulin ( $3-6 \times 10^4$  cpm). Radioactivity was measured in the blood, the volume of which in all the experiments described here was taken as 2.0 ml per 25 g body wt. and was expressed as a percentage of the injected radioactivity per total volume of blood. Each point is an average value obtained from three mice. (▲), Liposome-PE (control); (●), Liposome-PE (10 mol %)-dextran; (○), liposome-PE (18 mol %)-dextran; (Δ), liposome-PE (30 mol %)-dextran.

250 mg of dextran (uncoupled) and radio-labelled PE-liposomes (3 ml) and no significant effect was observed on the clearance of the latter as compared with PE-liposomes alone (data not shown).

Since the amount of dextran on the surface of liposome may be a determining factor for the retention in circulation, varying amounts of PE were taken for the preparation of dextran-liposome conjugates. The extent of dextran coupling is proportional to the number of free amino groups of PE present on the outer surface of liposomes. Thus an increase in the mol % of PE will reflect a corresponding increase in the amount of dextran molecules coupled with the PE liposomes. It was observed that the rate of disappearance of the conjugated liposome from the circulation decreases as the amount of PE in the liposome increases up to a critical concentration of 18 mol %, above which the rate of clearance remains unaltered. Therefore, this could be due to saturation of dextran binding sites on the surface of liposomes, resulting from a steric hindrance caused by the bound dextran molecules.

Differences in the survival of liposomal encapsulated marker in the circulation caused by coupling with dextran should reflect in the extent of uptake by liver and spleen. Tissue distribution of liposome entrapped  $\gamma$ -globulin at different time intervals is presented in table 1. The amount of radioactive marker taken up by the liver and spleen is greater for control liposomes than for dextran liposomes. In both the cases, the maximum uptake by the liver occurred 15 min after the administration of liposomes, after which there was a gradual decrease in the amount of radioactivity in the liver. This may be due to the possible lysosomal degradation of the protein marker in the liver.

Presumably dextran does not cross the cell membrane (Molteni, 1979) which could explain why proteins or haptens coupled to dextran remain in circulation for longer times. Though there are reports (Richter and Hedin, 1982) of dextran hypersensitivity on protracted use in a few patients, the use of clinical grade dextran may considerably reduce the chances of such toxicity in most of the individuals. A sensitivity test for dextran may be performed to avoid any possible side effect *in vivo*. However, the

Table 1. Distribution of [ $^{125}$ I]- $\gamma$ -globulin in mice tissues at different time intervals after injection of liposome entrapped [ $^{125}$ I]- $\gamma$ -globulin.

Liposomal lipid composition	Molar ratio of lipids	Time interval (min)	Liver	Kidney	Spleen	Lung
EL/Chol/PE	7:2:2	2.5	21.1 $\pm$ 2.1	0.8 $\pm$ 0.1	1.1 $\pm$ 0.1	1.1 $\pm$ 0.3
		5	30.8 $\pm$ 3.1	0.9 $\pm$ 0.2	1.2 $\pm$ 0.2	0.9 $\pm$ 0.3
		15	36.9 $\pm$ 2.5	1.2 $\pm$ 0.2	2.8 $\pm$ 0.4	0.8 $\pm$ 0.2
		30	21.0 $\pm$ 1.6	1.1 $\pm$ 0.1	3.1 $\pm$ 0.5	0.6 $\pm$ 0.1
		60	11.3 $\pm$ 1.2	0.9 $\pm$ 0.3	1.6 $\pm$ 0.2	0.5 $\pm$ 0.1
		120	4.7 $\pm$ 0.9	0.6 $\pm$ 0.1	0.9 $\pm$ 0.1	0.3 $\pm$ 0.1
EL/Chol/PE-dextran	7:2:1	2.5	18.3 $\pm$ 1.9	0.9 $\pm$ 0.2	0.9 $\pm$ 0.3	1.2 $\pm$ 0.4
		5	26.1 $\pm$ 1.7	1.1 $\pm$ 0.1	1.2 $\pm$ 0.2	1.1 $\pm$ 0.3
		15	30.8 $\pm$ 2.2	1.3 $\pm$ 0.3	2.3 $\pm$ 0.4	0.8 $\pm$ 0.2
		30	16.9 $\pm$ 0.9	1.4 $\pm$ 0.2	2.6 $\pm$ 0.4	0.8 $\pm$ 0.2
		60	9.2 $\pm$ 1.1	1.2 $\pm$ 0.3	1.8 $\pm$ 0.3	0.7 $\pm$ 0.1
		120	3.9 $\pm$ 0.8	0.9 $\pm$ 0.4	1.1 $\pm$ 0.1	0.5 $\pm$ 0.1
EL/Chol/PE-dextran	7:2:2	2.5	11.3 $\pm$ 1.1	0.8 $\pm$ 0.1	0.7 $\pm$ 0.2	1.3 $\pm$ 0.3
		5	15.6 $\pm$ 1.4	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2	1.4 $\pm$ 0.4
		15	20.1 $\pm$ 2.1	1.1 $\pm$ 0.1	2.0 $\pm$ 0.4	1.2 $\pm$ 0.1
		30	13.2 $\pm$ 1.3	1.6 $\pm$ 0.2	1.8 $\pm$ 0.6	1.0 $\pm$ 1.0
		60	8.3 $\pm$ 1.0	0.9 $\pm$ 0.4	1.3 $\pm$ 0.3	0.7 $\pm$ 0.2
		120	3.8 $\pm$ 0.7	0.8 $\pm$ 0.3	1.0 $\pm$ 0.1	0.5 $\pm$ 0.2
EL/Chol/PE-dextran	7:2:4	2.5	13.5 $\pm$ 1.5	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2	1.3 $\pm$ 0.1
		5	17.6 $\pm$ 2.1	1.0 $\pm$ 0.1	1.1 $\pm$ 0.2	1.2 $\pm$ 0.2
		15	22.7 $\pm$ 2.2	1.3 $\pm$ 0.3	2.1 $\pm$ 0.4	0.8 $\pm$ 0.2
		30	14.9 $\pm$ 1.3	2.0 $\pm$ 0.1	1.8 $\pm$ 0.6	0.7 $\pm$ 0.1
		60	10.8 $\pm$ 1.4	1.3 $\pm$ 0.2	1.6 $\pm$ 0.4	0.5 $\pm$ 0.2
		120	5.8 $\pm$ 1.1	1.0 $\pm$ 0.1	1.2 $\pm$ 0.2	0.5 $\pm$ 0.1

Values are mean per cent of injected [ $^{125}$ I]- $\gamma$ -globulin  $\pm$  S.D. Each group contained three mice.

optimal molecular size of coupled dextran and its role in diminishing the liposomal disappearance from blood remain to be determined.

Regardless of the mechanism by which dextran promotes the stability of liposome *in vivo*, dextran conjugated liposome should provide an effective means for controlled drug release over longer periods. Such controlled release may be of particular importance in diseases where the slow release of a drug is essential.

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## Influence of formamide on the thermal stability of DNA

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**Abstract.** The utility of formamide in the denaturation and renaturation of DNA has been examined. The melting temperature of duplex DNA is lowered by 0.6°C per per cent formamide. The depression of melting temperature is independent of the GC content. Formamide also increases the width of the thermal transition. Upto 30%, it does not affect the rate of DNA reassociation.

**Keywords.** DNA melting; formamide;  $T_m$ ; DNA base composition; DNA reassociation.

### Introduction

The optimum rate for DNA-DNA reassociation occurs at about 25°C below the melting temperature ( $T_m$ ), (Wetmur and Davidson, 1968; Britten *et al.*, 1974). The incubation temperature ( $T_i$ ) for reassociation, on account of its dependence on  $T_m$  varies with the GC content of the DNA. For DNA samples of GC content of 50% or less,  $T_i$  is within the normally accessible range for optical and hydroxyapatite chromatographic studies. For instance, the optimum temperature for monitoring reassociation of *Escherichia coli* DNA (GC content, 50%) in the presence of 1 M NaCl, is around 75°C. For DNA having 70% GC, in the presence of 1 M NaCl,  $T_i$  will be around 90°C. Incubation at such a high temperature often causes strand breakage and depurination of DNA. Hence the use of chemical agents whose presence lowers  $T_m$  without any specificity for base composition and does not hinder reassociation rates is advocated.

Formamide seems to be the choicest of the agents and has been in use more than any other compound both in solution and in filter hybridization studies. The effect of formamide on the destabilization of DNA duplex was first studied systematically by McConaughy *et al.* (1969) using *Bacillus subtilis* DNA (40% GC). According to them the rate of reduction of  $T_m$  was 0.72°C per per cent formamide. Later, Casey and Davidson (1977) reported a value of 0.63°C for *E. coli* DNA. In this communication we report studies on the effect of formamide on denaturation of DNAs with a wider variation in base composition.

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Abbreviations used: EDTA, Ethylenediamine-tetraacetic acid; RNA, ribonucleic acid; SDS, sodium dodecyl sulphate;  $T_i$ , temperature of incubation;  $T_m$ , temperature midpoint of melting.

## Materials and methods

### Bacterial strains

*Mycobacterium smegmatis* SN2, *E. coli* B and *Bacillus cereus* were obtained from our laboratory culture collection.

### DNA isolation

The cells were lysed by lysozyme-EDTA-sodium dodecyl sulphate (SDS), and the DNA was isolated by the phenol extraction procedure (Kirby, 1959). To achieve lysis of *M. smegmatis* cells sensitization of cells by pretreatment with glycine was necessary. Glycine (0.15 M) was added to the growing culture 2 h before harvesting. RNA was removed from the nucleic acid preparation by treatment with RNase A. In the case of *B. cereus*, a combination of RNase A and RNase T1 was used. After RNase digestion, sodium acetate was added (final concentration, 0.3 M) and the samples were extracted with (24:1) chloroform-isoamylalcohol (Marmur, 1961). DNA was precipitated with 2.5 volumes of ethanol, pooled and dissolved in 10 mM Tris pH 7.0, containing 1 mM EDTA and 10 mM NaCl.

### Reagents

Lysozyme, RNase A, RNase T1 formamide, Tris (hydroxymethyl) aminomethane and SDS were from Sigma Chemical Company, St. Louis, Missouri, USA. All other reagents were of analytical grade. Purification of formamide has been described elsewhere (Sadhu et al., 1984).

### Melting and reassociation of DNA

Thermal denaturation was done according to the procedure described by Mandel and Marmur (1968) using a Pye-Unicam SP700A spectrophotometer fitted with an electrical heating system. Absorbance was monitored at 270 nm because of the inherent absorbance of formamide at 260 nm. For reassociation studies, stoppered cuvettes containing DNA was heated by circulating water. Reassociation was initiated by the addition of prewarmed sodium-phosphate buffer and the decrease in absorbance at 270 nm was recorded for 1 h using Shimadzu ultra-violet recording spectrophotometer. In all the reassociation experiments  $T_i$  was maintained at 25°C below  $T_m$  at that condition. Results were plotted as suggested by Wetmur (1976).

## Results and discussion

### Melting of DNA in the presence of formamide

The  $T_m$  values for *M. smegmatis*, *E. coli* and *B. cereus* DNA in the absence of formamide were 90°C, 83.5°C and 78.5°C respectively (data not shown). Base compositions of these DNA species were determined from the equation (Mandel and Marmur, 1968):  $\Delta GC = \Delta T_m \times 2.44$  using *E. coli* DNA as the standard. Assuming the GC content of *E. coli* DNA as 50% (Marmur, 1961) the GC contents of *M. smegmatis* and *B. cereus* DNA were found to be 66% and 38% respectively.

$T_m$  values of each of these DNA samples in the presence of varying concentrations of formamide were determined. With increasing concentration of formamide  $T_m$  values of all the DNA samples were lowered. For each DNA sample, the  $T_m$  values were plotted against formamide concentration (figure 1). The slopes of all the lines were same, indicating that DNAs with varying GC content behave in an identical fashion in the presence of formamide. The drop in  $T_m$  with increasing concentration of formamide is linear and corresponds to 0.6°C per per cent formamide. As mentioned above, the DNAs employed in these studies varied in GC content from 38–66%. It is, therefore, possible that formamide does not interact with the bases in a differential manner.

For each melting profile, the standard deviation,  $\sigma$ , which is a relative measure of cooperativity, was calculated by the equation (Wada *et al.*, 1980):  $\sigma = (\Delta T_{14.1}^{85.9} - 0.6) \times 1.25$ . The results are presented in table 1. The value of  $\sigma$  increased with an increase in

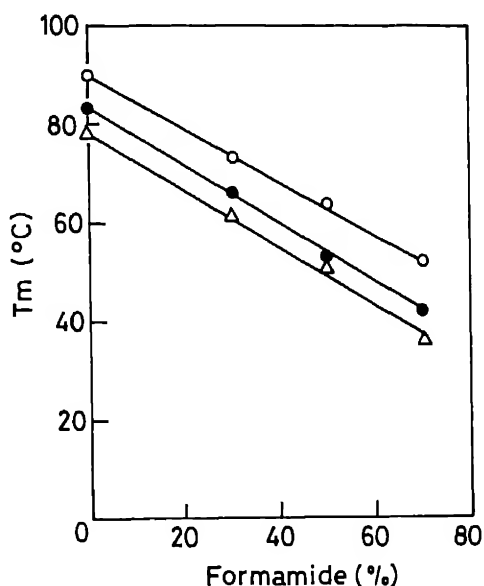


Figure 1. Effect of formamide on  $T_m$  of different DNA samples. (O), *M. smegmatis*; (●), *E. coli*; (Δ), *B. cereus*.

Table 1. Effect of formamide on  $\sigma$  value.

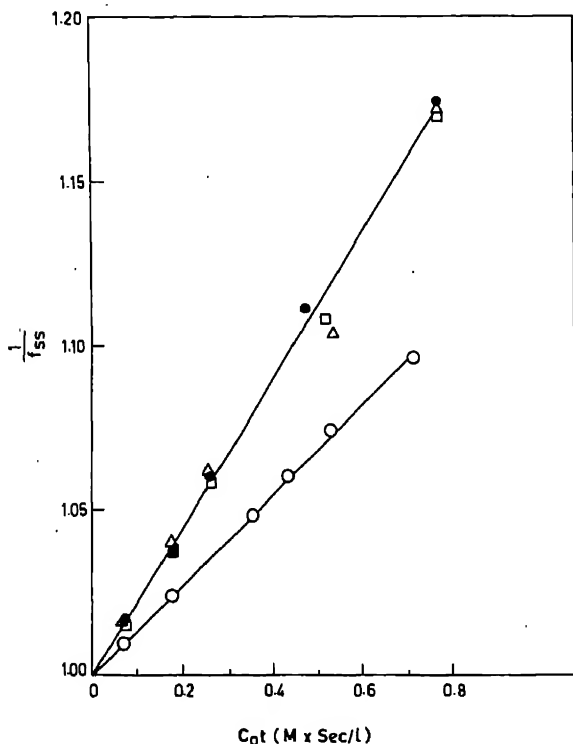
Source of DNA	$\sigma$ value			
	a	b	c	d
<i>B. cereus</i>	4.75	5.75	6.50	8.50
<i>E. coli</i>	4.50	4.80	5.50	8.62
<i>M. smegmatis</i>	4.00	5.25	5.50	10.25

(a), 0% formamide; (b), 30% formamide;  
(c), 50% formamide; (d), 70% formamide.

the formamide concentration. However, a quantitative correlation between GC content,  $\sigma$  values and formamide concentration could not be obtained. Cooperativity of the helix to coil transition in DNA is intensified by weakening the shielding effect of the ionic cloud (Wada *et al.*, 1980). The shielding effect can be altered by changes in ionic strength, dielectric constant etc. At a fixed ionic strength, dielectric constant of the medium is increased from 81 of the aqueous phase to 96 by the addition of formamide (1:1, v/v) (Record, 1967). This may be the cause of the observed increase of  $\sigma$  in the presence of formamide.

#### *Effect of formamide on the rate of reassociation*

We have examined the effect of formamide on the rate of DNA reassociation using *E. coli* DNA. With increasing concentrations of formamide, reassociation was carried out at lower temperatures to maintain a difference of 25°C between  $T_m$  and  $T_i$ . The results are shown in figure 2. Upto 30%, formamide showed no appreciable effect on the rate of reassociation. At higher percentages of formamide, the reassociation rate was reduced.



**Figure 2.** Effect of formamide on the rate of reassociation. Reassociation was carried out in the presence of 0.8 M phosphate buffer (pH 6.9). The  $T_i$  in the presence of 0, 15, 30 and 45% of formamide were 78°C, 69°C, 60°C and 51°C respectively.  $f_s$ , the fraction of single stranded DNA was calculated from the relation,  $1/f_s = (A_0 - A_\infty)/(A_t - A_\infty)$  where  $A_0$  is absorbance of entirely denatured molecules,  $A_\infty$  is absorbance at infinite time, and  $A_t$  is absorbance at time,  $t$ . ( $\Delta$ ), 0% formamide; ( $\square$ ), 15% formamide; ( $\bullet$ ), 30% formamide; ( $\circ$ ), 45% formamide.

The rate of reassociation in the presence of formamide can be influenced by the following factors:

(i) Increased accessibility of the complementary strands to each other due to an increase in the dielectric constant of the medium causing a decrease in the interstrand electrostatic repulsion.

(ii) Decreased bimolecular collisions due to the reduction of  $T_1$ .

A balance between these two opposing factors probably kept the rate more or less unaltered upto 30% formamide. Further increase in formamide concentration may give rise to the condition where the temperature factor overtakes the former causing the observed decreased in the rate of reassociation.

### Acknowledgements

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## Fluorescence studies on concanavalin-A

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**Abstract.** Using the lectin-concanavalin-A, the tryptophan fluorescence as a function of pH was studied. The pH dependent, fluorescence intensity changes were significantly higher when excited at 305 nm, than when irradiated at 280 nm. Only one tryptophanyl per monomer of concanavalin-A was available for oxidation by N-bromosuccinimide in the dimeric form at pH 4.9; no tryptophanyl could be oxidised in the demetallised dimer (pH 3.0) and native tetramer (pH 7.0). Based on this fluorescence data and the already known crystal structure data, it appears that tryptophanyl 88 in concanavalin-A may be selectively excited by 305 nm radiation.

**Keywords.** Concanavalin-A; fluorescence; selective excitation; 305 nm.

### Introduction

Concanavalin-A (ConA) is a lectin and a metalloprotein associated with a  $\text{Ca}^{2+}$  and a  $\text{Mn}^{2+}$  ion. It is a powerful agglutinating agent and binds specifically to  $\alpha$ -D-manno- and glucopyranosides and is the first lectin whose three dimensional crystal structure was determined (Reeke *et al.*, 1975). Physicochemical studies revealed that ConA can exist, depending on pH, as a dimer, a tetramer (Kalb and Lustig, 1968; McKenzie *et al.*, 1972) and also as a demetallised dimer in two different forms (Brewer *et al.*, 1983); under physiological conditions, ConA is tetrameric. The important role of aromatic residues in maintaining the tertiary and quaternary structure and also in the biological activity of proteins is now recognised (Sabesan and Harper, 1980). A recent study on ConA using radiotracer technique, reported that tryptophanyl and possibly tyrosyl residues are involved in the carbohydrate binding (Moore and Mudher, 1979). In this context, it is of interest to study the environment of tryptophanyl (Trp) residues using fluorescence as a probe. It was shown recently (Kuramitsu *et al.*, 1978; Rao *et al.*, 1981) that it is possible to selectively excite only one of the six Trp residues (Trp 108) in lysozyme by using 305 nm as excitation wavelength, instead of the usual 280 nm excitation. The present report deals with an investigation of the fluorescence of ConA excited at 305 nm and also at 280 nm, as a function of pH, and also in the presence of N-bromosuccinimide, which can oxidise Trp to a non-fluorescent oxindole compound.

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Abbreviations used: ConA, Concanavalin-A; Trp, tryptophanyl; NBS, N-bromosuccinimide; OD, optical density; Tyr, tyrosine.

## Materials and methods

ConA (Sigma Chemical Co., St. Louis, Missouri, USA highly purified) free of sugar and carbohydrates prepared from Jack beans by the method of Olson and Leiner (1967) was used. ConA free of cleaved fragments was prepared by the method of Cunningham *et al.* (1972) by treating the lectin with ammonium bicarbonate. N-Bromosuccinimide (NBS), urea and other chemicals used for making buffers are all of analytical grade. NBS was recrystallised from 90% ethanol.

The buffer systems used were: pH < 3.0: Gly-HCl; pH 3.8–5.4:  $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$ ; pH 6.0–8.5:  $\text{KH}_2\text{PO}_4-\text{Na}_2\text{HPO}_4$  (Diem and Lentner, 1971). An ionic strength of 0.3 was maintained in all the systems by the addition of NaCl. Stock buffer solutions were filtered through a G-3 sintered glass filter, and protein solutions were filtered through filters of 0.45  $\mu\text{m}$  pore size (Maxflow).

Optical density (OD) measurements were made with a Zeiss spectrophotometer, PMQ3, at room temperature. The number of tryptophans ( $n$ ) oxidised were calculated (Spande and Witkop 1967), using the equation:

$$n = \frac{\Delta\text{OD}_{280}}{\text{OD}_{280}} \times \frac{1.31 \epsilon}{5500} \quad (1)$$

where  $n$  is number of Trps oxidised per mol of ConA and  $\epsilon$  is molar extinction coefficient of ConA at 280 nm.

The concentration of the protein solutions were determined assuming a  $\epsilon_{280}^{1\%}$  of 12.4 at pHs below 7.0, and 13.7 at pHs above 7.0 (Kalb and Lustig, 1968; Yariv *et al.*, 1968); the protein concentration in the present study was  $\approx 10^{-6}$  M.

Fluorescence spectra were recorded with a Jasco FP-550 grating spectrofluorimeter at room temperature using two different excitation wavelengths, 280 nm and 305 nm. The intensity of emission ( $I_f$ ) with 305 nm excitation was weak compared to that with 280 nm excitation. The fluorescence intensity  $I_f$  at the emission maximum at both the excitation wavelengths (280 and 305 nm) was found to be linear in the concentration range used in the present study.

NBS solutions (1 mm) were added in small aliquots (25 or 50  $\mu\text{l}$ ) to ConA solutions and the fluorescence spectra were recorded 30 sec after the addition;  $I_f$  was corrected for dilution.

pH measurements were done with a Radiometer model pHM 26 pH meter.

## Results

The fluorescence spectra of ConA were recorded as a function of pH. The wavelength of maximum emission,  $\lambda$  (max, em), was found to be constant at 340 nm over the pH range 1.5 to 8.5, when irradiated with 305 nm, but  $\lambda$  (max, em) when excited at 280 nm was red shifted by 5 nm, from 333–338 nm, as the pH was raised to 7.0 (figure 1A). The pH dependence profile of fluorescence intensity ( $I_f$ ) at 335 nm (with 280 nm excitation) and at 340 nm (with 305 nm excitation), shown in figure 1B, has a maximum around pH 7.0. As the solution was rendered neutral, the  $I_f$  was enhanced by about 30%, when excited by 280 nm, but as much as a 90% increase was observed in fluorescence emission with

305 nm excitation (figure 1A). Above pH 7.0, in the alkaline region, a diminished fluorescence is a common feature of the two sets of data.

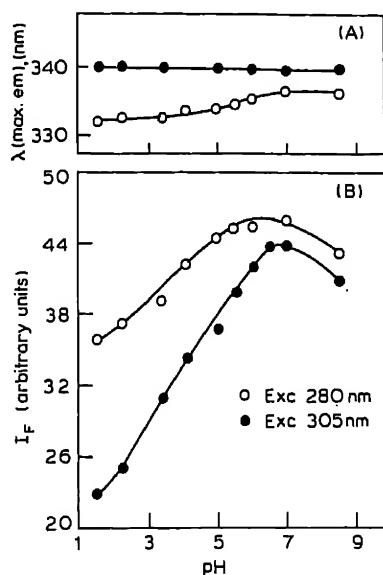


Figure 1.  $\lambda(\text{max, em})$ , (A) and fluorescence intensity;  $I_F$  (B) of ConA observed as a function of pH using 280 nm excitation (O)- and 305 nm excitation (●).

#### Oxidation of tryptophanyl residues using NBS

The course of oxidation of Trps by NBS in ConA was followed by the measurement of optical density at 280 nm as well as of fluorescence intensity as a function of added NBS at three pHs; the number of tryptophans oxidised in ConA was calculated using eq. (1) and the values obtained are tabulated in table 1. No tryptophan was available for oxidation, even after the addition of large amount of NBS, in demetallised dimer of ConA (pH 3.0) and also in the tetramer (pH 7.0). In the dimeric ConA (pH 4.0), however, two Trps out of the eight present were oxidised on addition of 12 mols of NBS per mol of ConA dimer; this oxidation, however, remarkably destabilized the protein, and turbidity was found to set in.

Table 1. Number of Trp of Con-A oxidised by NBS.

pH	ConA form	Number of Trp oxidised/dimer	
		No urea	8 M urea
3.0	Demetallised <sup>1</sup> dimer	0	8
4.9	Dimer	2	6
7.0	Tetramer	0	4

In view of the optical density data, NBS oxidation of dimeric ConA was followed by fluorescence only at pH 4.9. The decrease in fluorescence emission at 340 nm, as a function of mols of NBS added is depicted in figure 2A. There was an initial loss in fluorescence till 12 mols of NBS were added and thereafter  $I_f$  remained constant; the optical density data indicated that only two Trp residues per dimer were oxidised. However, the relative decrease in  $I_f$  in the 305 nm excited emission spectra is markedly higher ( $\approx 70\%$ ), than that in fluorescence excited at 280 nm ( $\approx 35\%$ ). Again the  $\lambda$  (max, em) with 280 nm excitation blue shifted by 10 nm (333–323 nm) on addition of NBS (figure 2B); there was, however, no change in the emission maximum (340 nm) of the spectrum excited at 305 nm.

#### *Oxidation of Trp residues in 8 M urea*

NBS oxidation of Trp residues in ConA was also studied in the presence of 8 M urea at three different pHs. The total number of Trps oxidised were calculated to be 6 and 4 per dimer, respectively in dimeric (pH 4.9) and tetrameric (pH 7.0) forms, while all the 8 Trps were oxidised in the demetallised dimer (pH 3.0) (table 1). On oxidation of Trps of ConA in 8 M urea solutions, the relative loss in fluorescence excited at 305 nm was similar to that observed at 280 nm excitation.

#### **Discussion**

Each monomeric ConA contains four Trp at positions 40, 88, 109, and 182, and seven tyrosines (Tyr) at 12, 22, 54, 67, 77, 100 and 176. Even so, the fluorescence behaviour of ConA, as any other class-B protein, is considered as that due to its Trp residues (Cowgill, 1963) because the quantum efficiency of Tyr is much less than that of Trp in proteins (Chen, 1967; Longworth, 1971). The increase in fluorescence intensity observed (figure 1B) as pH was raised from 1.5 to 7.0 is associated mainly with the ionisation of side chain carboxyl groups of aspartyl/ and glutamyl residues in ConA; carboxyl groups which are proximate to Trp are known to quench fluorescence and their ionisation removes the quenching (White, 1959; Gally and Edelman, 1961). The relatively larger pH-dependent increase in fluorescence intensity observed with 305 nm excitation, compared to that excited at 280 nm (figure 1A), suggests that tryptophans excited at 305 nm are in an environment different from most of the Trp excited at 280 nm (Kronman, 1976; Kuramitsu *et al.*, 1978; Rao *et al.*, 1981). Pelly and Horowitz (1976) have shown from fluorescence quenching studies of ConA by iodide ions that two Trps are near the molecular surface. Actually, analysis of three dimensional crystal structure data of ConA (Reeke *et al.*, 1975) clearly indicates that out of the four Trps, only two (at 88 and 182) are on the molecular surface, and, further, one of them (Trp 88) makes van der Waals contacts with two aspartyl residues, Asps 136 and 139. It appears then that Trp 88 is the most likely residue that is selectively excited by 305 nm radiation.

The decrease in fluorescence intensity above pH 7 in the alkaline region can be ascribed to the ionisation of tyrosyls; the phenoxide moieties act as 'energy sinks' for energy transfer from Trp to Tyr (Edelhoch *et al.*, 1967; Cowgill, 1963). It is to be noted that conformational changes that occur on changing pH may also affect the fluorescence intensity and  $\lambda$  (max, em) (Miller and Nwokedi, 1975).

The wavelength of emission maximum of Trp unlike of tyrosyls depends very much on the locale of the residues and  $\lambda$  (max, em) could be as low as 330 nm for Trp in the interior of the protein and as high as 350 nm for Trp at or near the molecular surface (Teale, 1960). In the present study, the  $\lambda$  (max, em) for 280 nm excitation was 333 nm at pH 1.5, and this was red shifted by about 5 nm on changing the pH to 7.0 and it is significant that over the pH range 1.5–8.5, the emission maximum of 305 nm excited fluorescence was unaltered at 340 nm.

NBS oxidation studies are expected to throw light on the relative exposure of tryptophanyl residues in a protein for, NBS readily oxidises those Trp at or near the molecular surface (Spande and Witkop, 1967). In fact, the extent of reaction of a protein with NBS in the presence of a denaturant, say 8 M urea, gives an estimate of the extent of denaturation (Hassing and Goldstein, 1972). Fluorescence intensity, as expected, decreased on addition of NBS at pH 4.9, and again, the decrease is significantly higher in the case of 305 nm excitation than with 280 nm irradiation (figure 2A). The decrease in  $I_F$  with 280 nm excitation was also accompanied by a blue shift in  $\lambda$  (max, em) (figure 2B) suggesting that a Trp near the molecular surface was oxidised.

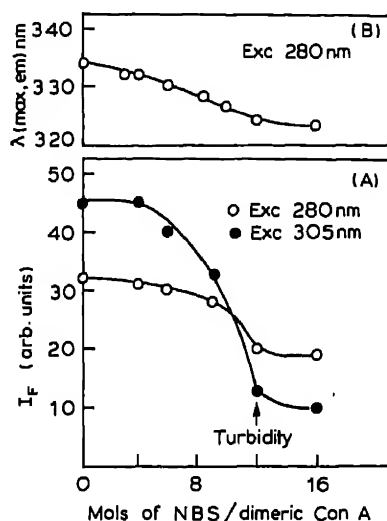


Figure 2. Changes in fluorescence intensity,  $I_F$  (A) and  $\lambda$  (max, em), (B) of ConA observed using 280 nm excitation (O) and 305 nm excitation (●) on addition of NBS.

The instability induced in the system leading to aggregation and turbidity, when one Trp per monomer of the dimeric ConA (pH 4.9) was oxidised, indicates clearly that this Trp has an important role in maintaining the tertiary and quaternary structure of ConA; aggregated ConA loses its biological activity (Hassing and Goldstein, 1972). The fact that Trp 88 is involved in monomer-monomer interactions in the formation of dimer (Reeke *et al.*, 1975) supports our suggestion of this residue to be the one selectively excited by 305 nm radiation.

ConA in the demetallised form is reported to be more easily denatured by 8 M urea than the native ConA containing metal ions (Agarwal and Goldstein, 1968) and further

this denaturation is irreversible (Pflumm and Beychok, 1974). Miller and Nwokedi (1975) showed that denaturation of ConA by urea is a two stage process; a low concentration of urea causes an increase in fluorescence intensity and at higher concentration ( $> 4$  M), quenching of fluorescence accompanied by a red shift in the  $\lambda$  (max, em) was observed. The present studies on ConA in the presence of 8 M urea showed that all the Trp residues are exposed and available for oxidation by NBS only at pH 3.0 in the demetallised dimer (table 1) and the fluorescence emission maximum (350 nm) shifts to that of tyrosyl residues (305 nm) as a result of oxindole formation. The unfolding of the lectin by 8 M urea at pHs 4.9 and 7.0 was found to be incomplete as evidenced by the fact that some Trp were still inaccessible for oxidation.

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## Degradation of the herbicide diclofop-methyl in soil and influence of pesticide mixtures on its persistence

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**Abstract.** The degradation of the herbicide [ $^{14}\text{C}$ ]-diclofopmethyl was investigated in moist parabrown podzol soil at 22°C. Radiochemical procedures were used to monitor the herbicide breakdown. The mineralization of the uniformly labelled aromatic ring was pursued by trapping the  $^{14}\text{CO}_2$  generated for 96 days. Diclofop-methyl was rapidly degraded in the soil with a half-life of about 8 days. The major breakdown product was the corresponding acid-diclofop, formed by a very rapid hydrolysis of the esterbond. With time the acid appeared to undergo strong binding or complexing to the soil. An intermediate 4-(2,4-dichlorophenoxy) phenol was recovered from the treated soil. Concentration of the phenoxyphenol increased upto 6 days followed by quick decline. Insecticide combination of parathion + Demeton-S-methylsulphoxide partially inhibited diclofop degradation in the soil.

**Keywords.** Diclofop-methyl; herbicide; degradation; pesticide mixtures; soil.

### Introduction

The herbicide diclofop-methyl, ( $\pm$ )-methyl 2-[4-(2,4-dichlorophenoxy) phenoxy] propionate, is the active ingredient (36 %) in the commercial formulation Illoxan®. This formulation is recommended in the Federal Republic of Germany as a post emergence herbicide for the control of annual grasses in sugar-beet crop. Studies of Anderson and Domsch (1978) with agriculturally feasible combinations of chemicals, applied at recommended concentrations have revealed that the degradation of a thiol carbamate herbicide diallate is partially inhibited by an insecticide chlorpyrifos in soil (personal communication). Such findings prompted us to look into pesticide combinations that can retard the degradation of diclofop-methyl in soil. Nevertheless in order to assess the persistence and eventual detoxification of diclofop-methyl, information is needed about such partial inhibition of degradation by simultaneously or successively added pesticides.

This paper describes the results of laboratory investigations designed to study the rate of degradation and influence of selected pesticides on both the rate and pattern of degradation of the herbicide diclofop-methyl in an agricultural soil.

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## Materials and methods

### Chemicals

Diclofop-methyl, diclofop and diclofop-methyl [ $^{14}\text{C}$ ]-uniformly labelled in the dichlorophenyl ring moiety were obtained from Farbwerke Hoechst AG, Frankfurt, Germany (specific activity 10  $\mu\text{Ci}/\text{mg}$ ; purity 97%). Acetone solution of the [ $^{14}\text{C}$ ]-diclofop-methyl was prepared (500  $\mu\text{g}/\text{ml}$ ) with a specific activity of 3.13  $\mu\text{Ci}/\text{ml}$  and used in the experiment. Other pesticides listed in table 1 and all reagents were purchased locally.

**Table 1.** Common and chemical names, chronology, formulations and uses of pesticides recommended for sugar-beet crop.

Common name	Commercial name	Chemical name	Formulations* and active ingredient	Uses**
Pyrazon	Pyramine	1-phenyl-4-amino-5-chloro-pyridazon	W.P. 65%	H
Lindane	Nexit	1,2,3,4,5,6,hexachloro-cyclohexane	E.C. 25.5%	I
Phenmedipham	Betanal	3-(methoxycarbonylamino-phenyl)-N-(3'-methyl-phenyl) carbamate	E.C. 16.2%	H
Diclofop-methyl	Illoxan	( $\pm$ )-methyl 2-[4-(2,4-dichlorophenoxy) phenoxy] propionate	E.C. 36%	H
Parathion + Demethon-S-methyl-sulphoxide mixture	E605-combi	0,0-diethyl-0-(4-nitro-phenyl)monothio-phosphate + 0,0-dimethyl-S-(2-ethyl-sulphonylethyl) thiophosphoric acid ester	E.C. 20% + 17.5%	I
Demethon-S-methyl-sulphoxide	Meta-systox	0,0-dimethyl-S(2-ethyl sulphonylethyl) thio-phosphoric acid ester	E.C. 25%	I

\* W.P. = Wettable powder; E.C. = Emulsifiable concentrate.

\*\* H = Herbicide; I = Insecticide.

### Soil

The soil used in the study was a parabrown podzol collected from an experimental field of the Institute. The soil had the following characteristics. Total carbon: 1.26%, total nitrogen: 0.11%, pH (KCl): 5.4, water holding capacity: 36.2 g water/100 g dry soil. The soil was passed through 2 mm sieve before use.

### Treatment with the herbicide

Two ml of acetone solution containing 1000  $\mu\text{g}$  [ $^{14}\text{C}$ ]-diclofop-methyl was mixed with 1000 g of soil to give an uniform concentration of 1 ppm. This approximately

corresponded to the recommended application rate of 3 litres formulation per hectare. Aliquots of treated soil (40 g) were weighed into ground-glass jointed flasks (100 ml capacity) and fitted with similarly jointed glass columns. Each column contained granular soda lime (8 g) held between 2 glass wool plugs, to trap evolved  $^{14}\text{CO}_2$  and another top layer of soda lime (4 g) to protect the  $^{14}\text{CO}_2$  trap from saturation with atmospheric  $\text{CO}_2$ . Air diffused through the column provided for an  $\text{O}_2$  concentration within the flasks equal to that of the ambient air. Flasks were incubated for 96 days in the dark at  $22^\circ\text{C}$ , with 12.5% moisture content.

#### *Treatment with pesticide mixtures*

Pesticides used in this study along with [ $^{14}\text{C}$ ]-diclofop-methyl, the quantities added as well as the sequence of application were patterned as per spray programme for sugar-beet crop, where this herbicide is used as a post emergence herbicide. Common and chemical names, chronology in which they are recommended for sugar-beet cultivation, formulations and uses of the pesticides included in the experiments are listed in table 1. Treatment and extraction schedules are detailed in table 2. All samples were incubated in the dark at  $22^\circ\text{C}$  and at stated intervals diclofop-methyl and its breakdown products were extracted and analysed.

Table 2. Treatment and extraction schedule.

days	Soil treatments				
	I	II	III	IV	V
0	—	1+2	1+2	1+2	1+2
33	3	3	3+4	3+4	3+4
47	—	—	—	5	5
75	—	—	—	—	6
105	—	—	—	—	—

Numbers 1 to 6 names of pesticides as in table 1 added to soil.

— No further treatment. Samples were taken for extraction.

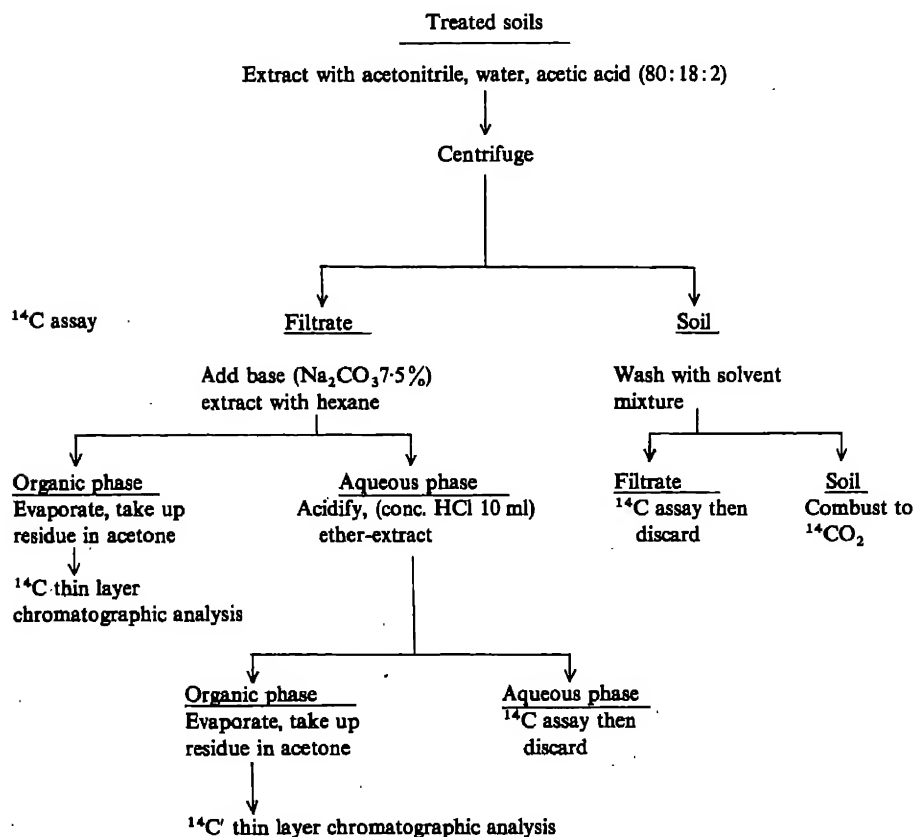
#### *Extraction and analysis*

The method employed to extract diclofop-methyl from the soil was similar to that reported by Smith (1977) with some modifications as outlined in flow-chart 1.

#### *Thin-layer chromatography*

Acetone extracts were applied to pre-coated thin layer chromatography plates (silica Gel 60F 254-E. Merck) and developed in the solvent system benzene: methanol: acetic acid (85: 10: 5 v/v). The radioactive areas on the developed plates were located by means of a scanner (Berthold and Friske, type II) and when more than one radioactive spots were present, they were scrapped off and measured directly in a toluene based scintillation solvent.

**Flow-chart 1.** Outline of procedures used for the extraction and analysis of [ $^{14}\text{C}$ ]-diclofop-methyl and its degradation products from soils. Thin layer chromatography solvent system: benzene:methanol:acetic acid (85:10:5).



### Soil combustion

Soil-bound residue was estimated by burning 1 g portion of the extracted soil with 300 mg cellulose powder in a packard sample oxidizer and trapping the evolved  $^{14}\text{CO}_2$  in Carbosorb®.

### Extraction of $^{14}\text{CO}_2$

The  $^{14}\text{CO}_2$  trapped by soda lime granules was released by acid treatment and quantitatively transferred to a mixture of ethanolamine:methanol (3:7 v/v) for analysis as described by Anderson and Domsch (1978).

### Radioactivity measurements

The radioactivity in the various solutions was measured using a liquid scintillation spectrometer (Nuclear-chicago Mark II). A commercial scintillation solution Unisolve I

(Zinsser, Frankfurt, Germany) was added to all samples containing water and for others toluene-based scintillation solution was used. The radioactivity measured was corrected for both background and for counting efficiency using the external standard.

## Results and discussion

### Degradation in soil

The mode of degradation of diclofop-methyl in soil for a period of 96 days is shown in figure 1. Diclofop-methyl disappeared rapidly from the soil. In between application and extraction (*Ca* 3 h) 35% was degraded and in 2 days approximately 90% of the applied 1 ppm herbicide was metabolized. Further degradation was slow and after 96 days, about 1.5% was still extractable. This is due to a certain amount of the applied herbicide getting bound to the humic fraction of the soil (Smith, 1977), which may not be available for degradation. Diclofop, ( $\pm$ )-2-[4-(2,4-dichlorophenoxy) phenoxy] propionic acid is known to be the first degradation product of this herbicide. Concentration of this acid increased in soil to *Ca* 70% within a day and thereafter gradually decreased to 5.5% at the end of the experiment.

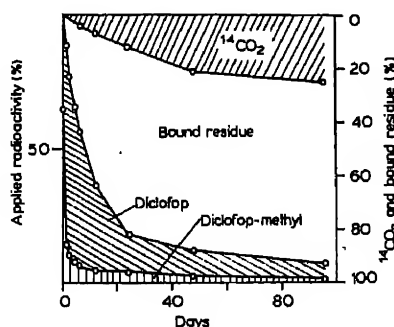


Figure 1. Degradation pattern of the herbicide [ $^{14}\text{C}$ ]-diclofop-methyl in parabrown podzol soil.

Using [ $^{14}\text{C}$ ]-diclofop-methyl, the degradation of the herbicide was studied further. As shown in figure 1, about 25% of the added herbicide was converted to  $^{14}\text{CO}_2$  in 96 days. Similar observations were made by Martens (1978). Since the radioactivity was present in the dichlorophenyl ring of the herbicide, it is clear that the herbicide was completely degraded in order to yield  $^{14}\text{CO}_2$ .

Most of the diclofop-methyl added (1 ppm) was converted into bound residues and was therefore resisting solvent extraction. At the end of the experiment as much as 65% of the added radioactivity was still unextractable and a maximum of 70% was detected as bound residues on day 24. While studying the aerobic degradation of diclofop-methyl in 3 prairie soils, Smith (1977) has also observed 40–60% resting in the soil. It was suggested that diclofop and the further metabolites can undergo binding or complexing to soil components making them resistant to extraction. However the

incorporation in to the microbial biomass is another way of rendering the chemical unextractable.

Diclofop-methyl has a half-life of about 8 days in the soil under the experimental conditions. It is reported that both diclofop-methyl and diclofop have herbicidal activity. Therefore concentrations of these two components were considered in determining the half-life (the length of incubation after which 50% of the applied herbicidal chemicals could no longer be extractable from the soil). At the half-life, about 5% of the herbicide was completely metabolized as evidenced from the evolved  $^{14}\text{CO}_2$  and 44% got converted into bound residues.

Degradation products could be resolved well from the diclofop-methyl in the chromatographic system used and a typical thin layer chromatography separation of the extracts after 2 days of incubation with the herbicide is shown in figure 2. At all sampling times the ether extract contained exclusively diclofop. However, the *n*-hexane phase which had only diclofop-methyl on day 0 showed two more radioactive peaks as the incubation continued. Judging from the mobility of the compounds in thin layer chromatographic plates and also comparing the observations of earlier reports (Martens, 1978; Smith, 1979), one metabolite with an  $R_f$  value of 0.43 could be identified as 4-(2,4-dichlorophenoxy) phenol. The identity of this chemical was later confirmed by comparing its  $R_f$  values with authentic dichlorophenoxyphenol in different solvent systems and also by co-chromatography. Table 3 reveals the  $R_f$  values

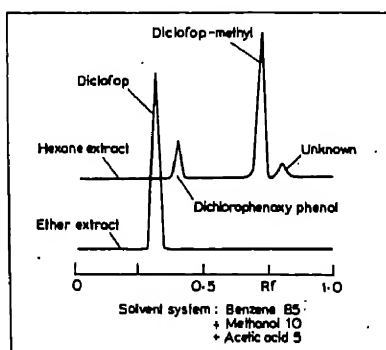


Figure 2. Radiochromatogram scanning of thin layer chromatographic plates showing diclofop-methyl and its degradation products.

Table 3.  $R_f$  values of compounds studied.

Compound	$R_f$			
	a	b	c	d
Diclofop-methyl	0.25	0.53	0.67	0.95
Diclofop	0.00	0.00	0.34	0.17
4-(2,4-dichloro- phenoxy) phenol	0.10	0.22	0.43	0.65

(a) Benzene-*n*-hexane (1:1); (b) benzene; (c) benzene-methanol-acetic acid (85:10:5); (d) benzene-methanol (10:1).

of compounds studied. No attempts were made to identify the metabolite(s) with an  $R_f$  value of 0.8. Since the concentration of these metabolites at any given time did not exceed 3% of the total activity, they are shown together with the diclofop-methyl fraction in figure 1.

Formation and disappearance of 4-(2,4-dichlorophenoxy) phenol in the soil is shown in figure 3. This substance was detected on the first day after treatment with diclofop-methyl. The concentration increased upto 2.5% of the added activity (day 6) and then came down to 0.4% in 96 days. Considering a slight but definite accumulation of the phenolic intermediate until day 6 and faster degradation thereafter, also, the lapse of 5 days between the appearance of extractable maxima for diclofop and its immediate intermediate, one can assume that the capability for the degradation of the phenolic compound in the soil appeared only after its formation.

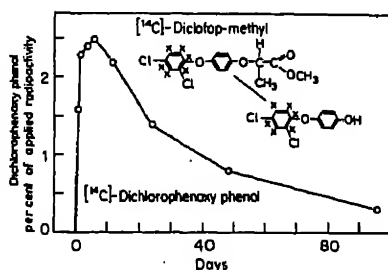


Figure 3. Formation and degradation of dichlorophenoxy-phenol in the soil.

#### *Effect of pesticide mixtures on diclofop-methyl degradation*

In the combinations and at the concentrations applied, none other than E605-combi had significant influence on either the rate or the pattern of degradation of diclofop-methyl in the soil (table 4). The insecticide mixture of parathion + demeton-S-methyl sulphoxide (E605-combi) slightly reduced the herbicide degradation. The partial inhibition was evident after the first sampling interval (day 75) and at the end of the experiment (day 105) there occurred about 20% inhibition in the degradation of diclofop-methyl with the consequent drop in  $^{14}\text{CO}_2$  formation. However thin layer chromatographic analyses of the soil extract displayed no difference in the pattern of degradation.

In order to confirm the effect of E605-combi on diclofop-methyl degradation, a separate experiment was set up wherein increasing concentrations of E605-combi was added to the herbicide treated soil (table 5). Between the concentration range of 2 to  $50 \mu\text{g} \times \text{g}^{-1}$  of the insecticides, the rate of degradation of diclofop-methyl was almost comparable to that of the control. However, soil-bound activity and  $^{14}\text{CO}_2$  production decreased with increasing concentrations of E605-combi, mainly due to the reduced rate of diclofop transformation. The mechanisms of interaction will be interesting study.

**Table 4.** Influence of pesticide mixtures on the degradation of [ $^{14}\text{C}$ ]-diclofop-methyl in soil.

Day	Distribution of radioactive compounds <sup>a</sup>	Treatments*				
		I	II	III	IV	V
33	Diclofop-methyl	48.0	48.5	49.5	—	—
	Diclofop	52.0	51.5	50.9	—	—
	Soil bound	0.0	0.0	0.0	—	—
47	CO <sub>2</sub>	8.8	7.6	7.8	7.7	—
	Diclofop-methyl	5.8	5.9	6.1	6.2	—
	Diclofop	31.9	30.0	30.9	32.2	—
	Soil bound	53.5	56.4	55.2	53.9	—
75	CO <sub>2</sub>	19.9	19.9	19.6	16.5	17.6
	Diclofop-methyl	3.1	3.3	3.4	3.7	3.8
	Diclofop	9.0	9.7	11.0	10.5	10.7
	Soil bound	68.0	67.0	66.1	69.3	67.5
105	CO <sub>2</sub>	23.6	24.5	24.0	19.7	23.2
	Diclofop-methyl	2.3	2.5	2.5	2.7	2.4
	Diclofop	6.8	7.3	7.0	8.2	8.5
	Soil bound	67.4	65.8	66.4	69.3	65.8

<sup>a</sup> Results are average from triplicates and presented as % of recovered radioactivity. Recovery  $99.2 \pm 2.2\%$  of added activity.

\* Refer to treatment schedule in table 2.

**Table 5.** Diclofop-methyl degradation in soil as affected by increasing concentration of E605-combi.

Day	Distribution of radioactive compounds <sup>a</sup>	Treatment with E605-combi ( $\mu\text{g} \times \text{g}^{-1}$ )				
		0	2	5	10	50
43	CO <sub>2</sub>	12.8	11.9	13.1	10.3	9.6
	Diclofop-methyl	2.7	2.7	2.8	2.7	2.7
	Diclofop	15.5	19.3	19.7	23.5	32.6
	Soil bound	69.0	66.1	64.4	63.5	55.1
85	CO <sub>2</sub>	21.1	19.5	22.2	17.4	18.0
	Diclofop-methyl	1.5	1.6	1.7	1.7	2.1
	Diclofop	5.3	7.1	8.3	9.6	20.0
	Soil bound	72.1	71.8	67.8	71.3	59.9

<sup>a</sup> Results are average from duplicates and presented as % of recovered radioactivity. Recovery  $98.4 \pm 7\%$  of added activity.

## **Acknowledgement**

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## The response of *Rhizobium meliloti* to L-methionine DL-sulphoximine

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**Abstract.** A strain of *Rhizobium meliloti* has been shown to be capable of growth in the presence of methionine sulphoximine concentrations at least two orders of magnitude higher than that required for the complete inhibition of glutamine synthetase activity. Neither the specific growth rate, nor the nutritional requirements of the organism were affected by methionine sulphoximine in the medium. *Rhizobium meliloti* appeared to assimilate ammonia via the glutamate dehydrogenase pathway during growth in the presence of methionine sulphoximine. This suggests that *Rhizobium meliloti* may have some regulatory mechanism controlling ammonia assimilation that is not present in other enterobacteria possessing similar enzymatic machinery.

**Keywords.** *Rhizobium meliloti*; methionine sulphoximine; glutamine synthetase; glutamate dehydrogenase.

### Introduction

The rhizobia have two enzyme systems for the assimilation of ammonia, the glutamate dehydrogenase (GDH) pathway, and the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. The rhizobia, however, possess two forms of GS (Darrow and Knotts, 1977). One form, GSI, is similar to *Escherichia coli* GS, while the other form, GSII, is unique to the Rhizobiaceae (Fuchs and Keister, 1980a, b). The exact role of GSII in rhizobial metabolism is unclear (Fuchs and Keister, 1980b; Ludwig, 1980). Methionine sulphoximine (MSX) is a potent inhibitor of GS (Meister, 1980). Most enterobacteria are inhibited by 1 mM MSX in the growth medium under conditions where the GS/GOGAT pathway is operative (Dendinger *et al.*, 1980; Steimer-Veale and Brenchley, 1974) but does not affect the growth of organisms using the GDH pathway (Brenchley, 1973). MSX resistance has been usually linked with glutamine auxotrophy (Masters and Hong, 1981) or bradytrophs (Miller and Brenchley, 1981). MSX resistance may also result from alterations in the GS protein (Miller and Brenchley, 1981). We have found that one strain of *Rhizobium meliloti*, is intrinsically resistant to MSX even under conditions where the GS/GOGAT pathway is generally operative. This resistance and its biochemical basis are described below.

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Abbreviations used: GDH, Glutamate dehydrogenase; GS, glutamine synthetase; MSX, methionine sulphoximine.

## Materials and methods

### Organism

*R. meliloti* 03 was isolated from effective nodules of fenugreek (*Trigonella foenum-graecum* L)

### Media

The organism was routinely maintained on medium containing 10 g mannitol, 400 mg  $K_2HPO_4$ , 200 mg  $MgSO_4 \cdot 7H_2O$  and 100 mg NaCl per liter, and where necessary, 15 g agar. The nitrogen source (nitrate, ammonia, or glutamate) was added to give 1 or 10 mM concentrations while glutamine was used at 0.5 or 5 mM concentrations. Glutamine was filter sterilized and added to sterile medium; MSX was added to sterile medium to give a final concentration equimolar to the nitrogen source.

### Cell-free extract

Cells used for enzyme assays or for preparation of cell free extracts were washed three times in 0.05 M Tris-HCl buffer (pH 7.2) containing 0.5 mM  $\beta$ -mercaptoethanol. For the preparation of cell free extracts, cells were frozen and then disrupted by sonication in the same buffer. The supernatant, obtained after centrifugation at 20,000 *g* for 20 min, was used for enzyme assays.

### Enzyme assays

Glutamine synthetase was assayed by the  $\gamma$ -glutamyltransferase assay (Shapiro and Stadtman, 1970) using cetyltrimethyl ammonium bromide in the assay mixture (Smith et al., 1980). Where used MSX was added to give 10–50  $\mu$ mol/ml assay mixture. GSI and GSII were assayed as the fractions stable and unstable at 50°C for 15 min respectively.

Glutamate dehydrogenase was assayed by the oxidation of NADH in cell free extracts.

Units of enzyme activity are defined as  $\mu$ mol  $\gamma$ -glutamyl hydroxamate formed or NADH oxidized/min/mg protein. Nitrate was estimated by the method of Garrett and Nason (1969). Ammonia was estimated by Nesslerization (Wriston, 1970). Protein was assayed by the method of Lowry et al. (1951).

### Gel electrophoresis

Polyacrylamide gel electrophoresis was performed on 5% polyacrylamide slabs of 1.5 mm thickness (Davis, 1964). Gels were stained for  $\gamma$ -glutamyl transferase activity.

## Results and discussion

Two forms of GS exist in *R. meliloti* 03, as in *R. meliloti* 41 (Fuchs and Keister, 1980b). These isoforms are separable on polyacrylamide gels (table 1). GSI, the heat stable isoform, has a lower electrophoretic mobility than the heat labile GSII, and its sensitivity to 60 mM  $Mg^{2+}$  varies, reflecting variations in the degree of adenylation

**Table 1.** Separation of *R. meliloti*, GSI and GSII on 5% polyacrylamide gel.

Sample treatment	Effect of GS activity band with relative electrophoretic mobility	
	0.18	0.46
None	+	+
50°C (15 min)	+	—
60 mM Mg <sup>2+</sup> <sup>a</sup>	+ / —	—
50 µM MSX	—	—

+ Presence or — absence of activity band.

<sup>a</sup> Incorporated into assay mixture.

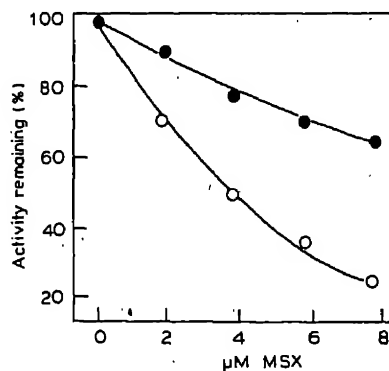
**Table 2.** The effect of 50 µM MSX on GSI and GSII in cell free extracts of *R. meliloti* 03 grown on 10 mM nitrate.

Enzyme	MSX	IU/ml GS	Inhibition (%)
GSI	—	0.0105	96
	+	0.0004	
GSII	—	0.1517	97
	+	0.0039	

(Darrow and Knotts, 1977). GSII in cell free extracts is completely inhibited by 60 mM Mg<sup>2+</sup>.

Both forms of GS in cell free extracts from 1 mM nitrate grown *R. meliloti* 03 are inactivated by MSX (table 1); over 95 % of the activity being lost at 50 µM concentration of MSX (table 2). This is similar to results obtained for *E. coli* GS (which is similar to GSI) by Ronzio *et al.* (1969). At lower MSX concentrations there is a slight, but reproducible, difference between the two enzymes (figure 1).

Although rhizobial GS is inhibited by such low concentrations of MSX, growth of this organism is not stopped even at 10 mM MSX. A hundred colonies of *R. meliloti* 03



**Figure 1.** Effect of MSX on GSI (O) and GSII (●) in cell free extracts of *R. meliloti* 03.

chosen at random, were transferred to a grid on a plate containing medium with 10 mM nitrate as nitrogen source. After the colonies were grown, they were replicated onto plates containing isonitrogenous nitrate, ammonia, glutamate or glutamine, with and without equimolar MSX, and the growth observed after 24 and 48 h (table 2). There was no difference in growth in the presence or absence of MSX, when glutamate or glutamine was the source of nitrogen. Glutamine is the product of the GS catalyzed reaction, while glutamate can compete with MSX for binding to the enzyme (Meister, 1980).

Every colony replicated onto medium containing MSX and nitrate or ammonia grew 24 h after colonies had developed on similar plates without MSX. Since every colony grew, this resistance to MSX is unlikely to be the result of mutation, and the 'lag' period probably reflects the time required for a change in the ammonia assimilation machinery. This hypothesis was supported by the fact, that once cells are grown on nitrate or ammonia and MSX, on further transfer to fresh medium, there is no difference in growth rate regardless of the nitrogen source or the presence or absence of MSX (data not shown). Cells transferred from MSX-containing medium to MSX-free medium showed normal GS activity and no delay in growth or alteration in growth rate (data not shown). It was noted that washed whole cells, grown on any nitrogen source in the presence of MSX did not possess detectable GS activity but did show GS activity when grown in the absence of MSX (data not shown).

Cell free extracts from washed cells grown on glutamate or nitrate, with or without equimolar MSX were assayed for GS and GDH; the results are shown in table 3. In glutamate grown cells there was GDH activity as well as GS activity in the absence of MSX and only GDH in the presence of MSX (table 4), while in nitrate grown cells GDH activity was observed only in the presence of MSX (table 4). The necessity for the induction of GDH may be one reason for the 'lag' observed on nitrate and ammonia but not on glutamate. In the enteric bacteria GDH is considered to be the major ammonia assimilatory enzyme during growth in excess ammonia (Smith *et al.*, 1975). However, in our organism, at growth temperatures below 30°C and at ammonia concentrations between 1–10 mM, most of the enzyme activity appears to be GSII (S. P. Rao, U. George and V. V. Modi, unpublished observations). Assays of other enzymes like asparagine synthetase and alanine dehydrogenase gave no significant activity.

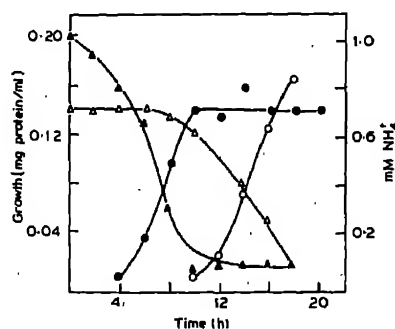
**Table 3.** Growth of *R. meliloti* 03 on various nitrogen sources in the presence or absence of MSX (unadapted cells).

Nitrogen source	MSX	Growth on plates after	
		24 h	48 h
Nitrate	—	++	+++
Nitrate	+	—	++
Ammonia	—	++	+++
Ammonia	+	—	++
Glutamate	—	++	+++
Glutamate	+	++	+++
Glutamine	—	++	+++
Glutamine	+	++	+++

**Table 4.** Levels of ammonia assimilatory in cell free extracts of *R. meliloti* 03 grown in the presence or absence of MSX.

Nitrogen source	Specific activity in extracts from cells grown					
	Without MSX			With MSX		
	GS I	GS II	GDH	GS I	GS II	GDH
Glutamate (10 mM)	0.149	0.289	0.047	—	—	0.103
Nitrate (1 mM)	0.009	0.329	—	—	—	0.095

Brenchley (1973) found that wild type *Klebsiella aerogenes* was resistant to 100 mM MSX during growth in high ammonia concentrations (35 mM), whereas GDH negative mutants were sensitive to 0.1 mM MSX. *Salmonella typhimurium* did not show similar MSX resistance (Steimer-Veale and Brenchley, 1974), although GDH was present in the organism (Dendinger *et al.*, 1980). GDH from most systems is reported to have a lower affinity for ammonia than GS (Stewart *et al.*, 1980). It was of interest therefore, to see if the rate of ammonia uptake increased during growth on MSX. Figure 2 shows that after the 'lag' period, neither growth nor ammonia uptake is markedly affected by MSX. This is particularly puzzling in that GDH is considered to be induced only in the presence of high intracellular ammonia concentrations. The uptake of ammonia in the presence of MSX is another difference between rhizobia and several other organisms. Many free living nitrogen fixers, including *Azotobacter vinelandii*, *Klebsiella pneumoniae* (Gordon and Brill, 1974) and several cyanobacteria (Orr and Haselkorn, 1982) export ammonia when fixing nitrogen in the presence of MSX. On the other hand, during the growth of *R. meliloti* 03 on nitrate, there was little difference in growth or extracellular ammonia levels (figure 3). In fact, when log phase cells, grown on nitrate, with or without MSX, were washed three times and resuspended in medium without  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (non-permissive for growth), more extracellular nitrite and ammonia were detected in medium containing cells grown in the absence of MSX (figure 4A,B). The presence or absence of MSX in the non-proliferating medium made no significant difference. The



**Figure 2.** Growth (○, ●) and ammonia uptake (△, ▲) by *R. meliloti* 03 growing on 1 mM  $\text{NH}_4\text{Cl}$  with (○), (△) or without (●), (▲) 1 mM MSX in the medium (unadapted cells).

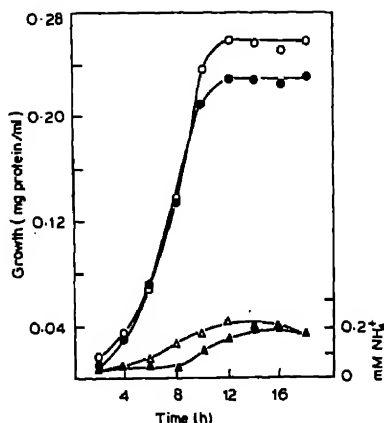


Figure 3. Growth (O, ●) and extracellular ammonia levels (Δ, ▲) during growth of *R. meliloti* 03 growing on 1 mM KNO<sub>3</sub> with (O, Δ) or without (●, ▲) 1 mM MSX (adapted cells).

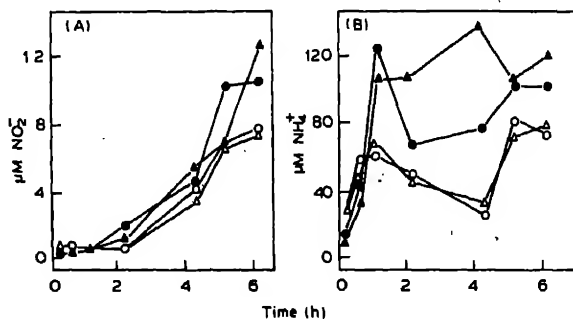


Figure 4. Nitrite (A) and ammonia (B) formation by resting cells of *R. meliloti* 03 growth with (O) or without (●) 1 mM MSX in 1 mM KNO<sub>3</sub>. Cells were incubated in the non-proliferating medium with (Δ, ▲) or without (O, ●) 1 mM MSX.

effect of MSX on nitrogen fixation was not determined since very poor asymbiotic nitrogen fixation has been obtained in this strain.

Apart from the presence of GSII in *R. meliloti* there appears to be little difference in the type of ammonia assimilatory enzymes present in this organism and in other enterobacteria (Dendinger *et al.*, 1980). GSII should make little difference to MSX sensitivity since this enzyme is also inhibited. Generally, MSX resistance is the result of changes in the enzyme such that the biosynthetic activity, but not the transferase activity of the enzyme is MSX insensitive (Miller and Brenchley, 1981), or organisms are mutated in other respects (Dendinger *et al.*, 1980). *R. meliloti* 03 shows no change in growth rates or growth requirements in the presence of MSX. The innate resistance of the organism to MSX, the apparent switch to GDH in the presence of MSX without corresponding increase in rates of ammonia uptake, and the lack of ammonia export even in resting cells, suggest that this MSX resistance of *R. meliloti* 03 is not the result of any special enzymatic capacity, but is the result of some special regulatory mechanism. The nature of this regulatory mechanism is yet to be determined.

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## Mechanistic studies on carboxypeptidase A from goat pancreas Part I: Role of tyrosine residue at the active site

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**Abstract.** Chemical modification of carboxypeptidase A<sub>1</sub> from goat pancreas with N-acetylimidazole or iodine led to loss of enzymic activity. This loss in activity could be prevented when chemical modification was carried out in the presence of  $\beta$ -phenylpropionic acid or substrate NCbz-glycyl-L-phenylalanine, thus suggesting a tyrosine residue at the active site. Chemical modification of tyrosine was confirmed by spectral and kinetic studies. While tyrosine modification destroyed peptidase activity, esterase activity of the enzyme remained unchanged thus indicating non-involvement of tyrosine residue in ester hydrolysis.

**Keywords.** Tyrosine; active site; goat carboxypeptidase; iodine; N-acetylimidazole.

### Introduction

Carboxypeptidase A from bovine pancreas was crystallised in 1937 (Anson, 1937) and its X-ray map was made available in 1968 (Lipscomb *et al.*, 1968). Enzyme was shown to contain one atom of zinc per mol, with histidine at 69 and 196 and  $\gamma$ -carboxylate ion of Glu 72 acting as ligand to zinc (Lipscomb *et al.*, 1969; Bradshaw *et al.*, 1969). Chemical modification studies confirmed these findings (Simpson *et al.*, 1963; Riordan and Vallee, 1963; Riordan *et al.*, 1967; Sokolovsky and Vallee, 1967; Riordan and Hayashida, 1970; Petra, 1971).

According to the current view of the bovine carboxypeptidase A catalysis, carboxylate ion can act as a general base catalyst and thus accelerate hydrolysis or it can act as a nucleophile generating an anhydride intermediate that would be labile to hydrolysis (Breslow and Wernick, 1977). Acyl anhydride has been demonstrated only in the case of ester substrate at  $-25^{\circ}$  to  $-40^{\circ}\text{C}$  (Makinen *et al.*, 1976). However, all attempts to detect anhydride with amide substrate or to trap it with alternate nucleophiles had failed (Kaiser and Kaiser, 1972). Modification of active site tyrosine group enhances the esterase activity while peptidase activity is lowered. Substitution of  $\text{Zn}^{2+}$  by  $\text{Hg}^{2+}$  also abolishes peptidase activity but increases esterase activity thus suggesting that the mechanism of catalysis in the two cases may not exactly be the same. Carboxypeptidase A from goat pancreas has been purified and resolved into two isozymes CPAG<sub>1</sub> and CPAG<sub>2</sub> (Dua and Ralhan, 1981a). The goat enzyme is free from some of the kinetic anomalies (Dua and Dixit, 1973) observed in the case of bovine enzyme (Vallee *et al.*, 1968; Davies *et al.*, 1968; Auld and Vallee, 1971) and hence may provide a simpler system to work with for the elucidation of the catalytic

mechanism. The goat enzyme differs from the bovine enzyme in N-terminus (Dua and Ralhan, 1981a) and product stabilisation (Dua and Ralhan, 1981b). Species differences have also been observed in the case of yeast carboxypeptidase which has no metal ion and has serine and histidine at the active site. Present studies deal with the chemical modification of goat pancreatic carboxypeptidase  $Ag_1$  which show a role for tyrosine residue at the active site.

## Materials and methods

N-acetylimidazole, NCbz-glycyl-L-phenylalanine, hippuryl-L-phenyllactic acid,  $\beta$ -phenylpropionic acid, glycyl-L-phenylalanine were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Iodine was obtained from British Drug House, Bombay.

Goat carboxypeptidase  $Ag_1$  was purified as reported earlier (Dua and Ralhan, 1981a). Carboxypeptidase A activity was determined colorimetrically using NCbz-glycyl-L-phenylalanine as a substrate (Folk and Gladner, 1958) while esterase activity was assayed spectrophotometrically using hippuryl-L-phenyllactic acid by the method of McClure *et al.* (1964). Protein concentration was determined colorimetrically by the method of Lowry *et al.* (1951) and spectrophotometrically by the method of Warburg and Christian (1941). Spectrophotometric studies were made using a Pye Unicam SP-500 spectrophotometer.

### Modification of carboxypeptidase $Ag_1$

Acetylation of goat carboxypeptidase  $Ag_1$  with N-acetylimidazole was performed by the method of Simpson *et al.* (1963). N-acetylimidazole (highly hygroscopic) was dissolved in dry benzene. Suitable aliquots of this solution were evaporated in tubes fitted with standard joint under vacuum and weighed. The reagent was prepared fresh each time. Acetylation was carried out in 50 mM borate buffer, pH 7.5 at 30°C in the presence of 300-fold molar excess of the reagent over the enzyme concentration for 45 min unless stated otherwise. Modified protein was desalted on Sephadex G-25 column (0.9 × 40 cm).

Iodination of goat carboxypeptidase  $Ag_1$  was carried out by the method of Vallee *et al.* (1963) with 50-fold molar excess of iodine over the enzyme concentration at 4°C in 20 mM sodium veronal-200 mM NaCl buffer, pH 7.5 for 60 min unless stated otherwise. Iodination was stopped by the addition of sodium thiosulphate solution. Modified protein was desalted by dialysis against 5 mM Tris-hydrochloride-200 mM NaCl buffer, pH 7.5 with several changes of the buffer. Iodine solution was prepared fresh each time by dissolving weighed quantity of iodine in 50 mM potassium iodide solution.

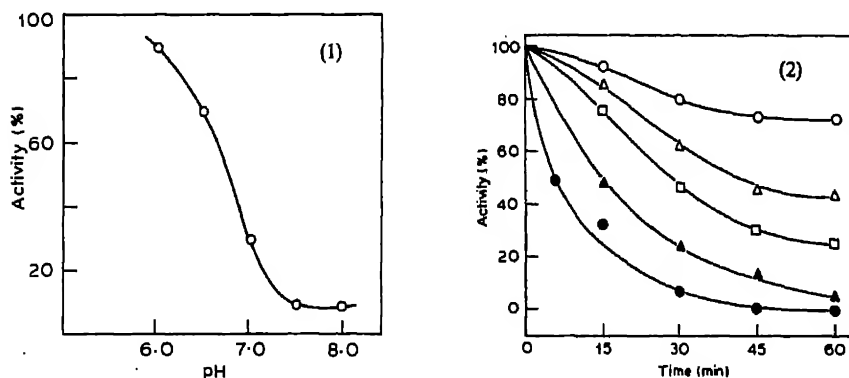
## Results and discussion

Treatment of a protein with iodine solution can lead to (i) iodination of tyrosine and/or histidine residues (ii) oxidation of cysteinyl sulphhydryl or tryptophan. However,

if the iodination is performed at pH 8.0 or below and in the presence of low concentration of the reagent, iodination may be confined to active site tyrosine. Selective iodination of tyrosyl side chains with loss of respective enzymatic activity has been shown in the case of *Escherichia coli*, asparaginase (Liu and Speer, 1977), bovine carboxypeptidase A (Riordan *et al.*, 1967) and D-3-phosphoglyceraldehyde dehydrogenase (Libor and Elodi, 1970).

Preliminary experiments indicated that iodination of tyrosyl residue of goat carboxypeptidase Ag<sub>1</sub> can be optimally carried out at pH 7.5 and 4°C (figure 1). However, this modification which led to loss of enzymic activity was reagent concentration and time dependent. At 20-fold molar excess of the reagent over protein, the enzyme lost 56% of its peptidase activity in 60 min while 100-fold molar excess led to complete loss of enzymic activity within 40 min (figure 2). Iodine is a potent non-specific inhibitor of enzymic activity. Hence that this loss of activity was due to modification and not due to inhibition, was established when exhaustive dialysis of the modified enzymes against 5 mM Tris-200 mM sodium chloride buffer, pH 7.5 for 24 h with four changes of the buffer failed to restore the enzymic activity.

The inactivation due to iodination was prevented when iodination was carried out in the presence of a substrate or its specific inhibitor. Only about 20% activity was lost



**Figures 1 and 2.** 1. Effect of pH on iodination of goat carboxypeptidase Ag<sub>1</sub>. One ml aliquots of the enzyme (protein: 0.2 mg/ml) were incubated for 60 min with 50-fold molar excess of iodine at 4°C at different pHs. Iodination was terminated by addition of 0.2 ml of 50 mM sodium thiosulphate and the reaction mixture dialysed against 5 mM Tris-200 mM sodium chloride buffer, pH 7.5 for 24 h and assayed for peptidase activity. Controls were run at respective pHs under similar conditions without iodine and per cent activity was calculated by taking control as 100. 2. Effect of concentration of iodine on the modification of goat carboxypeptidase Ag<sub>1</sub> as a function of time. Four ml aliquots of the enzyme solution (protein: 0.4 mg/ml) were incubated in the absence and presence of 10 (O); 20 (Δ); 30 (□); 50 (▲), and 100 (●) fold molar excess of iodine over the enzyme concentration in 20 mM veronal - 200 mM sodium chloride buffer, pH 7.5 at 4°C. Aliquots 0.5 ml were withdrawn at suitable time intervals and dialysed against 5 mM Tris-200 mM sodium chloride buffer, pH 7.5 for 24 h after terminating iodination by adding 0.2 ml of 0.05 M sodium thiosulphate. Controls were run under similar conditions without the addition of iodine. The per cent activity of the modified enzyme was plotted against time. The control value was taken as 100.

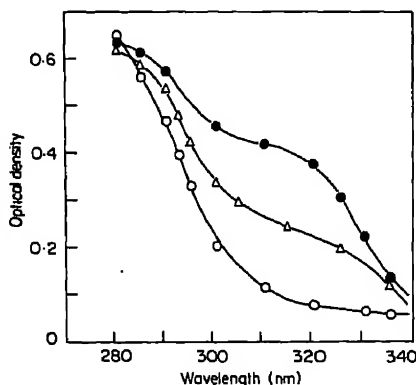
when iodination was done in the presence of  $\beta$ -phenylpropionic acid or NCbz-glycyl-L-phenylalanine as against 93 % in their absence (table 1), thus indicating that tyrosine being modified is at the active site and is protected by the presence of substrate or its inhibitor. Tyrosine modification was confirmed by comparing the spectra of the native and modified enzyme which showed an increase in absorption between 290–300 nm (figure 3). Such a shift in absorption from 278 to 290–300 nm has been attributed to tyrosine modification from model experiments (Hughes and Straessle, 1950).

N-acetylimidazole can acetylate  $\epsilon$ -amino group of lysine, or OH group of tyrosine. However, it has been shown that acetylation with N-acetylimidazole under controlled

**Table 1.** Effect of competitive inhibitor or substrate on the modification of goat carboxypeptidase A.

Treatment	Activity* (%)
Control	100
Modified with iodine in the absence of inhibitor/substrate	7.2
Modified with iodine in the presence of $\beta$ -phenylpropionic acid	78.9
Modified with iodine in the presence of NCbz-gly-L-phe	77.5
Modified with N-acetylimidazole in the absence of inhibitor/substrate	15
Modified with N-acetylimidazole in the presence of $\beta$ -phenylpropionic acid	96.3
Modified with N-acetylimidazole in the presence of NCbz-glycyl-L-phenylalanine	91.1

\*Two ml aliquots of the enzyme solution (protein: 0.15 mg/ml) were incubated in the absence and presence of 20 mM  $\beta$ -phenylpropionic acid or NCbz-gly-L-phe in 20 mM veronal – 200 mM sodium chloride buffer, pH 7.5 for 30 min at 30°C and were modified with 50-fold molar excess of iodine at 4°C for 60 min or with 300-fold molar excess of N-acetylimidazole for 45 min at 30°C in 50 mM borate buffer pH 7.5, desalted on Sephadex G-25, dialysed separately against 500 ml of 5 mM Tris-200 mM sodium chloride buffer, pH 7.5 for 18 h with four changes. Peptidase activity was determined in each case. The per cent activity left in the modified enzyme was calculated by taking the activity of unmodified enzyme as 100.



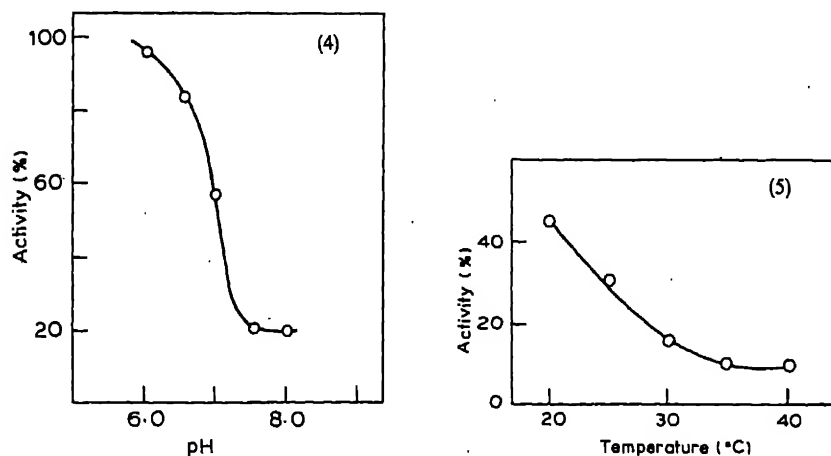
**Figure 3.** Ultra-violet absorption spectra of native and iodinated goat carboxypeptidase Ag<sub>1</sub>. Three ml of enzyme solutions (protein: 0.8 mg/ml) were modified under optimal conditions and dialysed against 5 mM Tris-200 mM sodium chloride buffer, pH 7.5. Ultra-violet spectra of modified and native enzyme were recorded at protein concentrations of 0.6 mg/ml. (O), Native enzyme; (Δ), iodinated enzyme for 30 min; (●), iodinated enzyme for 60 min.

conditions leads to selective acetylation of tyrosine residue with loss of respective enzymic activity of rabbit muscle aldolase (Schmid *et al.*, 1966; Pugh and Horecker, 1967), bovine carboxypeptidase A (Simpson *et al.*, 1963) and rapid loss of allosteric properties of rabbit muscle phosphofructose kinase (Chapman *et al.*, 1969).

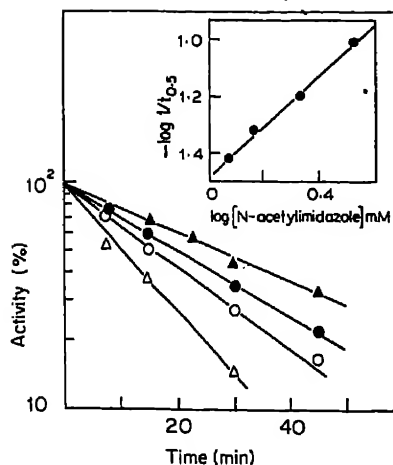
Preliminary investigation indicated that goat carboxypeptidase Ag<sub>1</sub> was also acetylated on treatment with N-acetylimidazole with concomitant loss of enzymic activity. Highest modification was obtained if the acetylation was carried out between pH 7.5 and 8.0 in 50 mM borate buffer at 30°C (figures 4, 5). However, pH 7.5 was chosen because of the better stability of N-acetylimidazole at this pH. Even at this temperature and pH, the extent of modification depended upon the concentration of the reagent used, *e.g.* incubation with 300-fold molar excess of N-acetylimidazole for 45 min led to 83% loss in activity as against 95% loss in activity in the same period when concentration of the reagent was raised to 450-fold molar excess (figure 6).

The loss in enzymic activity on treatment of carboxypeptidase Ag<sub>1</sub> with N-acetylimidazole was due to modification of the active site amino acid and not due to inhibition by the reagent because dialysis of the treated enzyme against 1 litre of 20 mM Tris-500 mM sodium chloride buffer, pH 7.5 for 24 h with several changes failed to restore the enzymic activity.

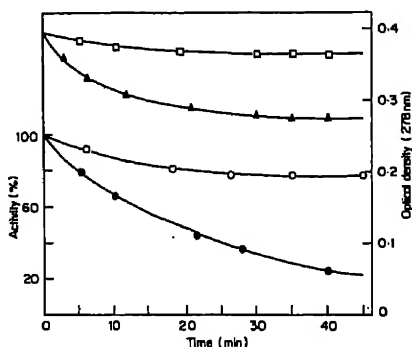
That treatment with N-acetylimidazole acetylates specifically tyrosine and not  $\epsilon$ -amino group of lysine was confirmed when ultra-violet absorption spectra of the native and acetylated enzyme showed a change in absorption only at 278 nm. Actually,



Figures 4 and 5. 4. Effect of pH on the modification of goat carboxypeptidase Ag<sub>1</sub> by N-acetylimidazole. Two ml aliquots of the enzyme solution (protein: 0.3 mg/ml) were incubated with 300-fold molar excess of N-acetylimidazole at 30°C for 45 min at different pHs, desalted on Sephadex G-25 column (0.9 × 40 cm), and their peptidase activity determined. Controls were run at the respective pHs under similar conditions without N-acetylimidazole. 5. Effect of temperature on the modification of goat carboxypeptidase Ag<sub>1</sub> by N-acetylimidazole. Two ml of enzyme aliquots (protein: 0.3 mg/ml) were incubated with 300-fold molar excess of N-acetylimidazole at different temperatures for 45 min at pH 7.5, desalted on Sephadex G-25 column (0.9 × 40 cm) and assayed for peptidase activity. Control was incubated under similar conditions without N-acetylimidazole. The per cent activity left in each sample was calculated by taking control as 100.



**Figure 6.** Effect of concentration of N-acetylimidazole on modification of goat carboxypeptidase  $Ag_1$  as a function of time. Eight ml aliquots of the enzyme solution (protein: 0.25 mg/ml) were incubated with 150 (▲); 200 (●); 300 (○), and 450 (Δ) fold molar excess of N-acetylimidazole in 50 mM borate buffer, pH 7.5 at 30°C. Aliquots were withdrawn at different time intervals, desalted on Sephadex G-25 column (0.9 × 40 cm) and assayed for peptidase activity. Control was incubated under similar conditions without N-acetylimidazole. The per cent activity left in each sample was calculated by taking control as 100. *Inset:* Determination of the order of inactivation by N-acetylimidazole.



**Figure 7.** Correlation of loss of peptidase activity and change in optical density at 278 nm on acetylation of carboxypeptidase  $Ag_1$ . Three ml of the enzyme solution (protein: 0.4 mg/ml) in 50 mM borate buffer, pH 7.5 was mixed with 0.05 ml of same buffer containing 75 or 300-fold molar excess of N-acetylimidazole over enzyme and incubated under optimal conditions. The absorbance of the enzyme solution containing different amounts of N-acetylimidazole were measured at 278 nm against the blank solution containing 3.0 ml of buffer and 0.05 ml of buffer containing 75 or 300-fold molar excess of N-acetylimidazole.

Aliquots (0.3 ml) were removed from reaction mixture at different time intervals, diluted with buffer to 1.0 ml and assayed for peptidase activity immediately. **A.** Modification in the presence of 75-fold molar excess of N-acetylimidazole. Peptidase activity (○); Change in optical density at 278 nm (□). **B.** Modification in the presence of 300-fold molar excess of N-acetylimidazole. Peptidase activity (○). Change in optical density at 278 nm (▲).

the decrease in optical density at 278 nm and loss in peptidase activity ran very much parallel (figure 7). Thus the enzyme acetylation could be easily followed spectrophotometrically at 278 nm. Treatment of acetylated enzyme with hydroxylamine led to deacetylation of the enzyme with restoration of the peptidase activity from 22 to 97.2 % of the original activity (table 2) and also restoration of native enzyme spectrum, thus supporting the view that tyrosine residue is being modified.

**Table 2.** Deacetylation of acetylated goat carboxypeptidase Ag<sub>1</sub> with hydroxylamine.

Sample	Activity <sup>a</sup> (%)
Native enzyme	100
Acetylated enzyme	22.3
Hydroxylamine treated acetylated enzyme	97.2

<sup>a</sup> One ml of 2 M hydroxylamine solution (pH 7.5) was added (to a final concentration of 330 mM) to 5.0 ml of acetylated protein (0.5 mg/ml) in 50 mM borate - 200 mM sodium chloride buffer, pH 7.5. The reaction mixture was incubated for 15 min at 30°C and excess of hydroxylamine was removed by passing through Sephadex G-25 column (0.9 × 40 cm) and assayed for peptidase activity.

The fact that the tyrosine residue being modified is at the active site was established when the presence of  $\beta$ -phenylpropionic acid or NCbz-glycyl-L-phenylalanine in the reaction medium protected the enzyme from modification. Loss in enzyme activity in their absence was 85 % but in their presence it was only 3.7 % and 8.9 % respectively (table 1). Thus binding of specific inhibitor or substrate at the active site protects the enzyme from acetylation which again suggests that tyrosine being acetylated is at the active site.

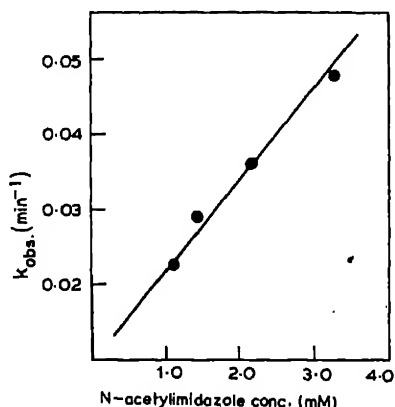
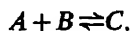
#### *Kinetics of modification with N-acetylimidazole*

Goat carboxypeptidase Ag<sub>1</sub> was incubated with various concentrations of N-acetylimidazole in 50 mM borate buffer, pH 7.5 at 30°C. Aliquots were withdrawn at regular intervals, desalted on Sephadex G-25 column, and assayed for residual peptidase activity. The inactivation process follows pseudo-first order kinetics at N-acetylimidazole concentrations used (figure 6) since the modifying reagent concentration greatly exceeded that of the enzyme.

The number of molecules of N-acetylimidazole reacting per mol of the enzyme were calculated by plotting  $-\log(t_{0.5})^{-1}$  vs. the log of N-acetylimidazole concentration (Marcus *et al.*, 1976; Hollenberg *et al.*, 1971). A slope of 0.9 was obtained (figure 6 inset) indicating that one molecule of N-acetylimidazole reacted with one mol of the enzyme leading to its inactivation. When observed pseudo-first order rate constants ( $k_{\text{obs}}$ ) were



plotted against concentration of N-acetylimidazole (figure 8) a linear plot was obtained suggesting a one step reaction of the following type (Strickland *et al.*, 1975):



**Figure 8.** Direct plot of observed pseudo-first order rate constant *vs.* concentration of N-acetylimidazole. Observed pseudo-first order rate constants ( $k_{obs}$ ) were calculated for 1.09 mM, 1.45 mM, 2.18 mM and 3.27 mM concentration of N-acetylimidazole from the semilogarithmic plot of inactivation of goat carboxypeptidase  $\text{Ag}_1$  (figure 6). The  $k_{obs}$  *vs.* concentration of N-acetylimidazole was plotted and the number of steps involved in the inactivation process determined.

#### *Effect of tyrosine modification on esterase activity*

The goat carboxypeptidase  $\text{Ag}_1$  has both esterase and peptidase activities. So the question arises whether active site tyrosine is needed for the hydrolysis of both type of substrates or only one type. Hence the enzyme was acetylated under optimal conditions for different time intervals, samples withdrawn, desalted on Sephadex G-25 and assayed for esterase activity. No significant change in esterase activity was observed (table 3). So it can be concluded that tyrosine is not involved in ester hydrolysis. This aspect of enzymic properties was also confirmed by modifying tyrosine residue with iodine. While peptidase activity was almost completely lost on iodination, no significant change in esterase activity was observed.

Such an involvement of a tyrosine residue in bovine carboxypeptidase A catalysed hydrolysis of acyldipeptides has been demonstrated by iodination (Riordan *et al.*, 1967), acetylation (Simpson *et al.*, 1963), nitration (Sokolovsky and Vallee, 1967), diazotization with diazo-*p*-arsanilic acid (Klesov and Vallee, 1977) or diazotized 5-amino 1-H tetrazole (Cucni and Riordan, 1978). However, in these cases modification of the tyrosine residue led to a significant increase in the esterase activity. No such increase in the esterase activity on modification of a tyrosine residue of goat enzyme has been observed. Hence it is likely that the active site of the enzyme from the two sources differ in detail.

**Table 3.** Effect of tyrosine modification on esterase activity of goat carboxypeptidase Ag<sub>1</sub>.

Time of incubation (min)	Activity <sup>a</sup> (%)
15	92.2
30	97.3
45	95.7
60	93.33
90	92.52
120	90.7

<sup>a</sup> Eight ml aliquot of the enzyme solution (protein: 0.6 mg/ml) was incubated with 300-fold molar excess of N-acetylimidazole under optimal conditions. Aliquots were drawn after 15, 30, 45, 60, 90, 120 min and subjected to gel filtration in Sephadex G-25 column (0.9 × 60 cm). Control was subjected to similar conditions without N-acetylimidazole. Activity of the control was taken as 100.

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## Role of ascorbic acid in metabolism of rat testis and epididymis in relation to the onset of puberty

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**Abstract.** The metabolism of ascorbic acid, cholesterol, serum testosterone level and activities of  $3\beta$  and  $17\beta$  hydroxysteroid dehydrogenases were studied in testis and cauda epididymis of prepubertal, pubertal and postpubertal (5, 15, 30, 45, 55 and 60 day old) rats. The data showed that serum testosterone levels and  $3\beta$  and  $17\beta$  hydroxysteroid dehydrogenases were increased with the age. The ascorbic acid metabolism was found to be stabilized in testis at day 30 being comparable with the adult, whereas a spurt in its metabolism occurred by day 45 and a significant depletion in ascorbic acid content in relation to the passage of the first wave of spermatozoa through cauda epididymis. The results of this study clearly elucidate that ascorbic acid is involved in metabolism of testis and epididymis in developing postnatal rats, in relation to the increasing demands for attaining a stable hormonal milieu, and the onset of puberty and the passage of the first wave of spermatozoa, *via* the formation of its free radical monodehydroascorbic acid and charge transfer complex mechanism.

**Keywords.** Ascorbate metabolism; steroidogenesis; rat testis; epididymis.

### Introduction

Ascorbic acid (AA) is a biologically active reductant whose metabolic significance in animal, human tissue and biological fluids has been extensively elucidated (Chinoy, 1978; Chinoy *et al.*, 1982). Its involvement during steroidogenesis is *via* the formation of its free radical, monodehydroascorbic acid (MDHA) (Agrawal and Laloraya, 1977; Chinoy *et al.*, 1978) which if coupled with steroids, *viz.*, pregnenolone and testosterone might produce progesterone or their active metabolites in the rat corpora lutea or testis. That AA influences the activity of  $3\beta$  hydroxysteroid dehydrogenase in normal toad testis and synergizes with luteinizing hormone in hypophysectomized toad testis *in vitro* has also been suggested (Biswas, 1971). The combined role of trophic hormones, C-AMP, prostaglandins and AA in the regulation of adrenal and gonadal steroidogenesis, each acting at various levels in hypothalamo-hypophyseal-adrenal/gonadal axis is reported (Datta and Sanyal, 1978). An interrelationship exists between AA and sex hormones in AA synthesizing tissues and steroidogenic organs of male and female rats (Chinoy and Rao, 1979; Chinoy *et al.*, 1979; Chinoy and Seethalakshmi, 1978).

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Abbreviations used: AA, Ascorbic acid; MDHA, monodehydroascorbic acid; ASG, ascorbigen; AAU, AA utilization; AA-MM, AA macromolecule; AA-FR, AA free radical; TAA, total AA; DHA, dehydroascorbic acid; RAA, reduced AA; GSH, glutathione; HSD, hydroxysteroid dehydrogenase.

However, the ascorbate metabolism in testis and epididymis in relation to the onset of puberty and the possible role of AA and its mechanism of action during steroidogenesis in gonad is not yet fully understood. Hence, the present study was undertaken in postnatal rats ranging in age from 5–65 days.

### Materials and methods

Healthy male albino rats (*Rattus norvegicus*) of Holtzman strain of different age groups (5, 15, 30, 45, 55 and 65 day old) were used for the experiments. They were maintained on a standard diet (Hindustan Lever Ltd., Bombay) and water was provided *ad libitum*. The animals were caged in an air conditioned animal house at a temperature of 20°C and exposed to 12–14 day light hours. The animals of each group were sacrificed accordingly and their testis and cauda epididymis were used for biochemical estimations. The testis and cauda epididymis were excised, blotted free of blood and were weighed on a torsion balance to the nearest milligram.

#### Ascorbic acid

The levels of free ascorbate (free AA), ascorbigen (ASG) AA utilization (AAU) and AA macromolecule (AA-MM) complex were carried out according to the method of Chinoy *et al.* (1976) and expressed as mg/g fresh tissue wt.

Ascorbic acid free radical (AA-FR) forming special peroxidase activity was assayed by the method of Chinoy (1973). The units were activity/g fresh tissue wt/20 min.

Total AA (TAA), dehydroascorbic acid (DHA) and reduced AA (RAA) were estimated by the method of Roe and Kuether (1943). The concentrations were expressed as  $\mu\text{g}/100\text{ mg}$  fresh tissue wt.

Glutathione (GSH) levels were estimated by the modified method of Grünert and Phillips (1951) and expressed as  $\mu\text{g}/100\text{ mg}$  fresh tissue wt.

The total cholesterol was assayed in testis and epididymis by the method of Pearson *et al.* (1953) and the concentrations were expressed as  $\text{mg}/100\text{ mg}$  fresh tissue wt. The histochemical localization of  $3\beta$  and  $17\beta$  hydroxysteroid dehydrogenases (HSDs) were carried out by the method of Tso and Lofts (1977).

*In vitro* study on sliced testicular tissue was done by the method of Dorrington and Fritz (1975). The testicular slices (200 mg) from post-pubertal rats were incubated with 4 ml of Krebs' Ringer bicarbonate buffer with 0.1% glucose (KRBG, pH 7.4). Pregnenolone (500  $\mu\text{g}/\text{ml}$ ) was added to the medium with or without supplementation of 0.001 M AA (176  $\mu\text{g}/\text{ml}$ ). The tissue slices were incubated for 2 h at 31°C in a continuous atmosphere of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  until analyzed for testosterone levels.

#### Testosterone levels by RIA

Blood of different age groups of rats were collected by cardiac puncture at 11.30 in the morning in every case so as to avoid diurnal fluctuations in hormonal levels. The testosterone levels were determined in serum/medium by the method of Castro *et al.* (1974) at the Institute for Research in Reproduction, Bombay. The antiserum was supplied by the World Health Organization. It was raised in sheep against testosterone-

3-(O-carboxymethyl) Oxime-bovine serum albumin. It cross-reacted with DHT to 14% without any significant reaction with other steroids. The sensitivity of the assay was 3–5 pg/tube. The intraassay and interassay coefficients of variation were 6 and 7% respectively and the levels were expressed as ng/ml medium/serum.

For all quantitative studies, a minimum of six replicates were done and their results were statistically analyzed using student's 't' test.

## Results

Six groups of rats ranging in age from 5–65 days were used. Each group contained 15–30 animals.

### Ascorbate turnover pattern

The levels of free AA were significantly ( $P < 0.001$ ) higher in testis of 45 day old rats followed by 15 and 55 day old ones. In 30 and 65 day old rats, the levels were more or less the same and least in 5 day old rats. In cauda epididymis, highest AA was found in 45 day old rat followed by 30 and 55 and least was in 65 day old rats (table 1).

The bound ASG was not detectable in testis of 45 and 55 day old rats, whereas in 65 day old ones, it was significantly ( $P < 0.001$ ) more than in the other three age groups. In cauda epididymis, the ASG levels were declined progressively with age from 45–65 day old rats. In 25 day old ones, it was not detectable (table 1).

The rate of AAU was high and almost the same in 5 and 15 day old rats. It progressively enhanced from day 30 onwards to 55 day old rat testis and subsequently declined abruptly in 65 day old rat to the level found in 30 day old ones. In cauda

**Table 1.** Showing the levels of free AA, ASG, AAU, AA-MM complexing and the activity of AA-FR forming special peroxidase in testis and cauda epididymis of pubertal (5, 15, 30 day old), pubertal (45 and 55 day old) and postpubertal (65 day old) rats.

Parameter	Tissue	Age in days					
		5	15	30	45	55	65
Free AA*	Testis	1.16±0.13	5.89±0.9	3.2±0.1	7.6±0.3	4.12±0.2	3.0±0.2
	Cauda	—	—	14.2±1.2	19.5±0.8	11.4±0.7	6.03±0.01
ASG*	Testis	0.42±0.04	0.43±0.07	0.33±0.1	ND	ND	0.79±0.12
	Cauda	—	—	ND	5.8±0.4	2.32±0.5	0.91±0.07
AAU*	Testis	45±0.9	53±2	18±0.3	31.5±1.6	49.6±3.2	17.8±2.3
	Cauda	—	—	37±2.1	46.5±1.3	35.5±3.8	22.6±1.3
AA-MM*	Testis	ND	ND	ND	ND	ND	1.39±0.4
	Cauda	—	—	ND	ND	ND	4.21±0.5
AA-FR**	Testis	28±2.6	6.14±0.35	3.87±0.2	8.12±0.1	14.8±0.8	3.4±0.2
	Cauda	—	—	9.3±0.8	13.2±0.3	11.6±0.6	18.6±0.2

Values are mean ± S.E.

\* mg/gm fresh tissue weight.

\*\* Activity/gm fresh tissue weight.

ND = Not detectable.

epididymis the highest AAU was obtained in 45 day old rats, followed by 30 and 55 day old ones. The least was observed in cauda epididymis of 65 day old group (table 1).

The net bound complexing of AA (AA-MM complex) was not detectable in both testis and cauda epididymis of any age group rats, except in the 65 day old ones. The AA-FR peroxidase activity in the testis showed the same trend as AAU, whereas in cauda epididymis, the enzyme activity increased in 45, 55 and 65 day old rats than in 24 day old animals. The latter (65 day old rats) had the highest activity (table 1).

#### *TAA, DHA, RAA and GSH*

The levels of TAA were less in the testis of 5-55 day old rats as compared to 65 day old ones, whereas, in cauda epididymis the levels were higher in 30 and 65 day old rats than in 45 and 55 day old ones which had comparable values. The DHA decreased in 55 day old rat testis and was significantly ( $P < 0.01$ ) higher in 65 day old rats as compared to others. In epididymis, DHA increased in 55 and 65 day old rats in comparison to 30 day prepubertal ones, but was least in 45 day old rats. RAA was not detectable in the testis of prepubertal and pubertal rats except 30 day old ones, but increased in 65 day old rats. In epididymis, a decrease at 45 days and nondetectable levels at 55 days were observed, but the highest was again in 65 day old rats (table 2).

The GSH levels were increased throughout in 45, 55 and 65 day old rat testis than in 5 and 30 day old groups. In cauda epididymis, it declined at 45 and 55 days and then increased in 65 day old rats (table 2).

#### *Cholesterol*

The cholesterol levels in the testis and epididymis were found to be higher in pubertal rats (45 day old rats) and subsequently a marked depletion occurred ( $P < 0.001$ ) at 65 days. In 5 and 15 day old rat testis, the cholesterol was significantly ( $P < 0.001$ ) lower in comparison to other groups (table 2).

**Table 2.** Showing the contents of TAA, DHA, RAA, GSH and cholesterol in testis and cauda epididymis of different age groups of rats.

Parameter	Tissue	Age in days					
		5	15	30	45	55	65
TAA*	Testis	0.11 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.08 ± 0.001	0.74 ± 0.01
	Cauda	—	—	0.72 ± 0.13	0.52 ± 0.16	0.59 ± 0.16	0.76 ± 0.01
DHA*	Testis	0.26 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.39 ± 0.01
	Cauda	—	—	0.39 ± 0.05	0.28 ± 0.04	0.62 ± 0.04	0.42 ± 0.01
RAA*	Testis	ND	ND	0.04 ± 0.001	ND	ND	0.38 ± 0.01
	Cauda	—	—	0.33 ± 0.05	0.24 ± 0.03	ND	0.38 ± 0.01
GSH**	Testis	12.5 ± 0.72	15.2 ± 1.5	13.2 ± 0.58	17.7 ± 0.58	16.5 ± 1.02	16.2 ± 1.01
	Cauda	—	—	32.6 ± 3.26	24.5 ± 1.50	29.3 ± 3.1	47.8 ± 6.0
Cholesterol***	Testis	0.14 ± 0.01	0.18 ± 0.002	0.25 ± 0.01	1.07 ± 0.01	0.52 ± 0.08	0.32 ± 0.01
	Cauda	ND	ND	0.30 ± 0.04	0.43 ± 0.02	0.36 ± 0.03	0.28 ± 0.01

Values are mean ± S.E. \* mg/gm fresh tissue weight. \*\* µg/100 mg fresh tissue weight. \*\*\* mg/100 fresh tissue weight. ND = not detectable.

### Hormonal levels

Significantly ( $P < 0.02$ ;  $P < 0.001$ ) higher levels of circulating testosterone were found in adult rats as compared to those of other groups. The testosterone production *in vitro* by postpubertal rat testicular slices were found to be comparatively more ( $P < 0.5$ ) in the medium containing pregnenolone + ascorbic acid than the medium having pregnenolone alone (table 3).

The activities of  $3\beta$  and  $17\beta$  HSDs in rat testis were increased from pubertal to adult stage as is evident from the decreased duration of the incubation period and the increased intensity of the formazan granule deposition (table 3).

**Table 3.** Showing the circulating levels of testosterone, activities of  $3\beta$  and  $17\beta$  HSDs of testis in prepubertal, pubertal and postpubertal rats and influence of AA in rat testicular testosterone synthesis *in vitro* using pregnenolone.

Group	Testosterone (ng/ml medium)		Testosterone (ng/ml serum)	$3\beta/17\beta$ HSDs	
	Pre. alone	Pre. + AA		Incubation time (h)	Intensity
Prepubertal	—	—	$0.22 \pm 0.03$ (6)	5	+ (less)
pubertal	—	—	$0.60 \pm 0.10$ (6)	4.5	++ (moderate)
Postpubertal	$6.68 \pm 0.5$ (4)	$8.02 \pm 0.7^*$ (4)	$2.37 \pm 0.74$ (5)	$3\frac{1}{2}$	+++ (highest)

Values are mean  $\pm$  S.E. Pre. = Pregnenolone. + = Intensity of formazan granule deposition. The number in parenthesis indicates 'n' value. \*  $P < 0.5$

### Discussion

The ascorbic acid metabolism in the 30 days old rat testis was found to be more or less stabilized since majority of the parameters involved were almost similar to the adult rats. This period in the postnatal development of the rat seems to be a crucial one involving rapid structural changes and metabolic activity in preparation for the onset of puberty. The observations of the present study are supported by those of ultra-structural changes in rat cauda epididymis and vas deferens (Chinoy and Asok Kumar, 1982; 1983) as well as those on localization and assay of AA in reproductive tissue of male rats (Chinoy *et al.*, 1983 a, b; 1984).

The ascorbic acid levels showed a spurt on day 45 in both testis and epididymis with the onset of puberty, concomitant with a decline in the levels of bound ascorbic acid. It implies that around day 45 an active mobilization of the bound ascorbate to its free form occurs for its subsequent rapid utilization, which increased with age, reached maximum levels by day 45 and thereafter declined in both tissues of 65 day old rat. This might be due to the increased requirements of AA for the animal which is attaining reproductive maturation. In 55 day old animals with the passage of the first wave of spermatozoa through the epididymis, the bound ascorbic acid was also significantly



depleted (in epididymis) suggesting AAU by sperms for their active metabolism and motility in agreement with our earlier data (Chinoy and Buch, 1977; Chinoy, 1978; Chinoy *et al.*, 1982).

That AA is involved in steroidogenesis of the gonads has been reported (Agrawal and Laloraya, 1977; Datta and Sanyal, 1977; Chinoy *et al.*, 1982). The present data supports these findings. A significant increase in circulating testosterone levels in postpubertal rats was accompanied by alterations in cholesterol levels in testis and Leydig cell activity. Chinoy *et al.* (1983) reported that AA deficiency in guinea pigs caused an androgen deprived effect to the target organs by probably influencing Leydig cell/or by affecting testosterone synthesis in testis. The scorbutic condition also reduced the fertilizing ability of guinea pigs and the contractility of vas deferens. These data reveal that AA is essential for the maintenance of the structural and the functional integrity of androgen target reproductive organs (Chinoy *et al.*, 1983 a, b; 1984). The primary action seems to be at the testicular level and the other changes were secondary manifestations as a consequence of the primary effect (Chinoy *et al.*, 1984).

The 65 day postpubertal rat testis and cauda epididymis showed AA-MM complexing for the first time during post-natal development. Similarly the enzymatic AAU as evidenced by AA-FR peroxidase activity occurred in testis only on day 65 but was initiated earlier in the epididymis of 45 day old rats. The synthesis of TAA was more in 65 day old rat than in other age groups. It is known that the levels of ascorbic acid in testis and epididymis are significantly greater than in liver, where it is synthesized. Moreover, tissues possessing a higher rate of metabolic activity have a correspondingly higher turnover of AA (Chinoy, 1978; Chinoy *et al.*, 1982) and a profound sex difference exists in its levels in rat tissues where the males possess higher concentration as compared to females.

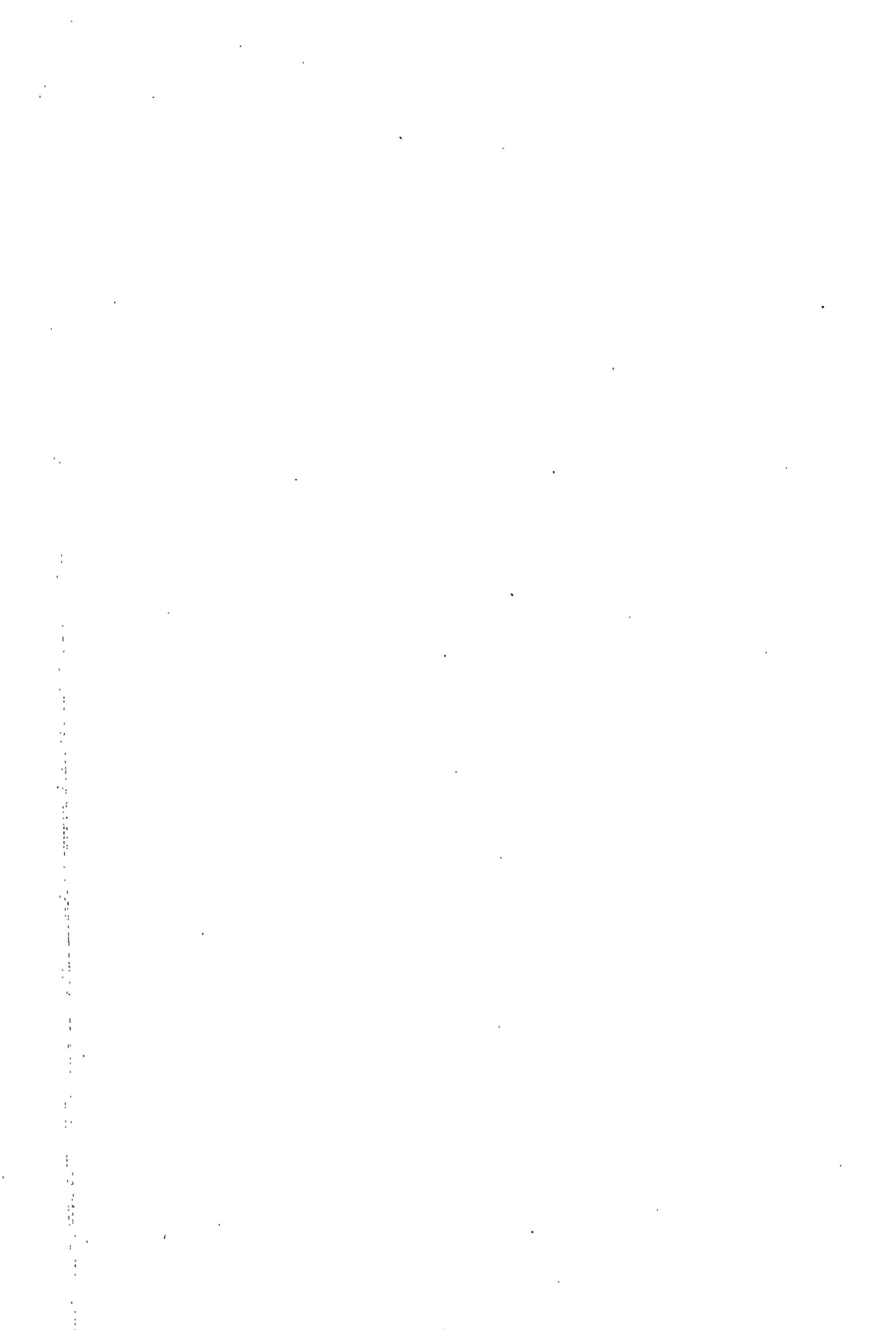
It is evident from the present data that AA acts in testis and epididymis *via* the formation of its free radical MDHA, which is eventually fully oxidized to DHA. The MDHA by virtue of possessing an unpaired electron is a much more powerful reducing agent than AA itself and is known to be involved in several oxido-reduction reactions, enzyme activation and potentiates the anabolic action of steroid in rat testis and epididymis (Kutsky, 1973; Lewin, 1976; Chinoy, 1978; Chinoy *et al.*, 1982) by forming charge transfer complexes with macromolecules (Chinoy *et al.*, 1978). Therefore, the mechanism of action of ascorbic acid is *via* the paramagnetic electron flow from MDHA and charge transfer complexing with macromolecules during postnatal development in rats. MDHA is an additional source of electron energy together with the energy obtained through the conventional breakdown of ATP in corroboration with our earlier data (Chinoy *et al.*, 1983a, b; 1984).

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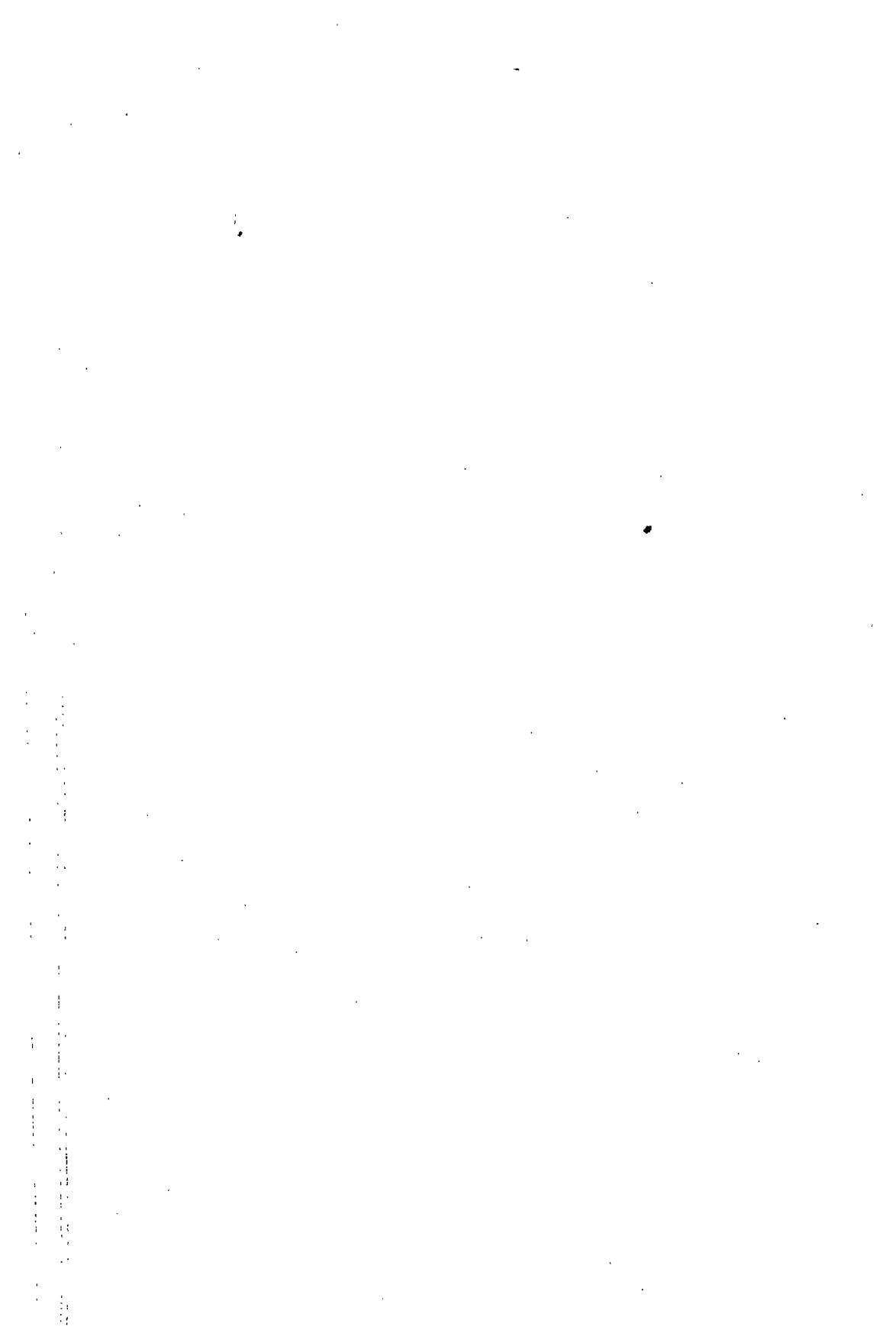
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## Foreword

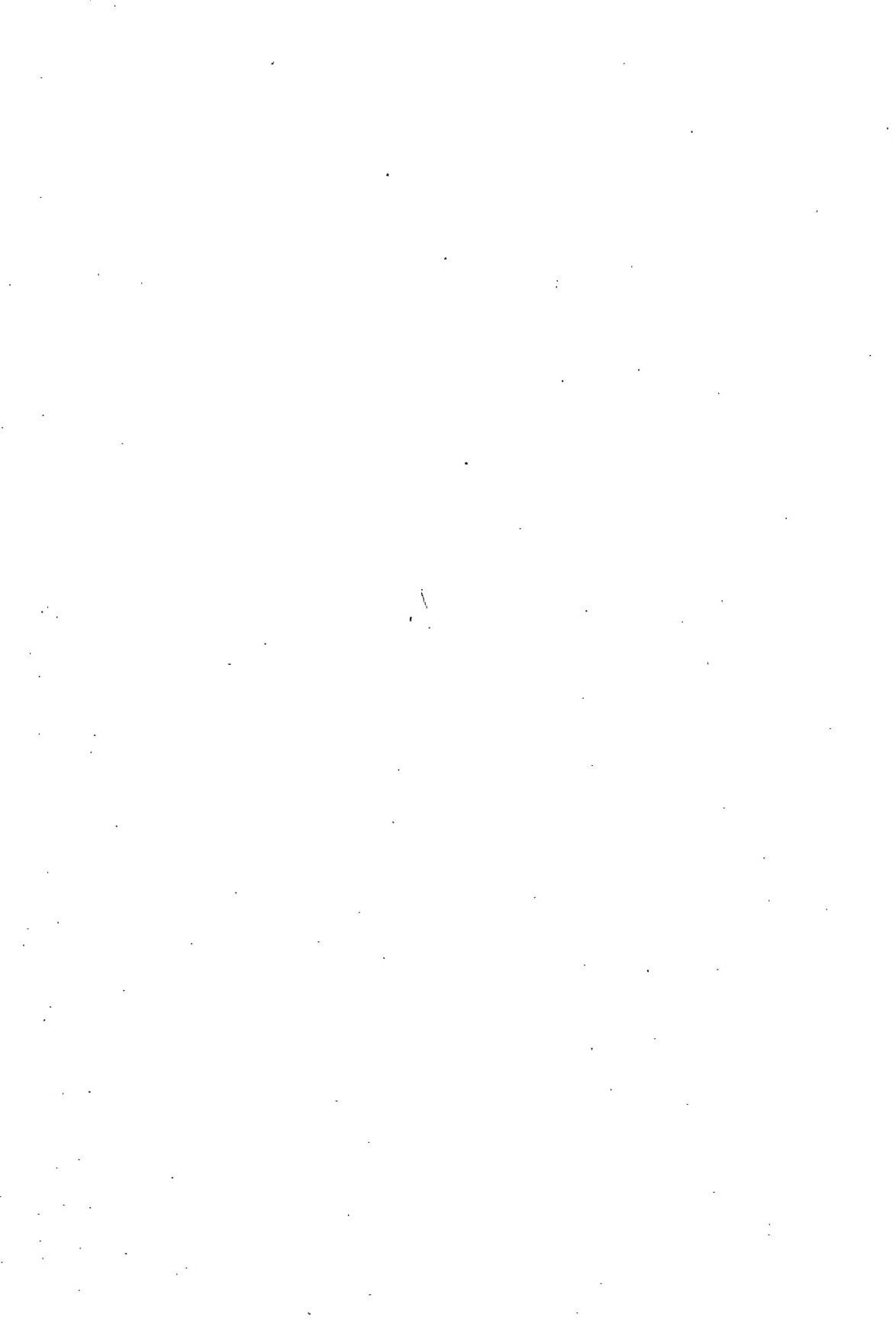
The present volume represents the Proceedings of a Symposium held under the Indo-US Subcommission and cooperation on Science and Technology at the Indian Institute of Science, Bangalore between December 12-16, 1983. This meeting was jointly sponsored by the Indian Council of Medical Research, New Delhi and the Department of Health and Human Services, NIH, Bethesda, USA.

The subject matter of the symposium 'Blastocyst Research' continues to receive attention from biologists of varied expertise and the present volume essentially updates the information available currently on different fronts. One of the primary objectives of the symposium was to see how relevant the findings using different animal models on blastocyst survival during pre- and post-implantation period is to our understanding of this subject in primates including man. The symposium covered a vast array of subjects ranging from ultrastructural investigations on blastocyst attachment to techniques involved in blastocyst retrieval; *in vitro* fertilization in the hamster and the monkey; isolation of trophoblast specific proteins secreted into the culture medium by pig and sheep blastocysts under culture; physiological and biochemical aspects of uterine receptivity to blastocyst implantation, metabolism of preimplantation blastocysts; on post-coital agents and menses inducing drugs; studies on pregnancy specific vitamin carrier proteins; on the regulation of chorionic gonadotropin secretion in primates and finally on the hormonal requirements during periimplantation period in the rodent and the primate.

One of the highlights of the meeting was a General Discussion of all the participants on the final day of the symposium with a view to enumerate the present state of the art and bring out the lacunae in our knowledge as well as recommend further lines of research of possible use in contraceptive development. I am thankful to Dr. M. R. N. Prasad for agreeing to be the Moderator of this Round Table Discussion.

As one of the convenors of the meeting, I would like to express my sincere appreciation of the Indian Academy of Sciences, Bangalore to have agreed to publish the proceedings of this meeting as a 'Supplement' to the Journal of Biosciences. My thanks are also due to my colleagues Drs. A. J. Rao, C. S. Sheela Rani, G. S. Murthy and P. R. Adiga for help rendered in organizing the symposium.

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## Hormonal requirement for ovum implantation

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**Abstract.** Hormonal requirement for ovum implantation varies among the species of animals. The methods attempting to clarify the requirement in each species may be classified as follows: (i) hormonal replacement therapy after removal of the pituitary or/and the ovaries, (ii) hormonal treatment after reduction of specific hormones by its antiserum *in vivo*, (iii) close observation of hormone secretion pattern in early pregnancy, (iv) examination of physiological conditions where implantation is delayed and analyze the hormone levels and the receptivity of the target tissues, and (v) examination of effects on hormone levels and the receptivity of target tissues of drugs which interfere with implantation. The reported results indicate that both progesterone and estrogen are needed for implantation in rats, mice, and Mongolian gerbils; in other species of animals progesterone alone may be sufficient to induce implantation, although synergistic effect of estrogen appears to be seen in some species such as in the rabbit. It remains to be determined whether the blastocysts of those animals that need only progesterone for implantation have greater ability to produce estrogen than the blastocysts of the animals that need both progesterone and estrogen. Control mechanism of secretion of progesterone and estrogen for inducing implantation may be different in various species. It has been suggested that both leutropin and follicle stimulating hormone are needed for pre-implantation estrogen secretion in the rat, whereas only follicle stimulating hormone is needed in the mouse. In the species where the obligatory delay in implantation is observed, neuroendocrine mechanisms are reported to be involved in controlling the pituitary-ovarian function that causes a delay in implantation.

**Keywords.** Implantation; gonadotropins; progesterone; estrogen.

### Introduction

There exists a species variation in the hormonal requirement for ovum implantation. Because of inadequate data on hormonal requirement for ovum implantation for each species of animals, there is a tendency to discuss hormonal requirement in general. In an attempt to delineate the existing species difference, hormonal requirement for ovum implantation will be reviewed separately for each species of animals.

### Rats

In the rat both progesterone and estrogen are required to prepare the uterus for implanting blastocysts. Progesterone alone does not induce implantation (Canivenc *et al.*, 1956; Cochrane and Meyer, 1957). Progesterone secretion prior to day 5 of

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Abbreviations used: LH, Leutropin or luteinizing hormone; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin, LHRH, LH-releasing hormone.



pregnancy is controlled by prolactin (Cutuly, 1941; Lyons *et al.*, 1943). It is not clear as to the degree of leutropin (LH) involvement in the luteotropic complex during the preimplantation period. Implantation takes place in the evening of day 5 in the rat (day 1 = sperm positive day). The ovarian steroids necessary for the preparation of the uterus for implanting blastocysts complete their output by 0100 h on day 5. Although gonadotropin secretion for preimplantation increase of estrogen takes place during day 4, it is not clear whether LH alone or both LH and follicle stimulating hormone (FSH) are needed for estrogen secretion. Lactation stimulates prolactin secretion and reduces gonadotropin secretion. Since prolactin is a luteotropic hormone and a new set of corpora lutea is formed at the postpartum ovulation that occurs within 24 h after parturition, progesterone secretion is elevated during lactation (Eto *et al.*, 1962; Tomogane *et al.*, 1969; Yoshinaga *et al.*, 1971). Serum level of progesterone during lactation is proportional to the size of suckling litter. The larger the size of suckling litter, the higher is the prolactin level and thus the higher is the progesterone level. Reduction in estrogen secretion in lactating rats nursing a large litter appears to be due to reduced secretion of gonadotropin(s) since injection of human chorionic gonadotropin (hCG) (Yoshinaga, 1976) or FSH (Raud, 1974) abolished a delay in implantation in lactating pregnant rats.

Although the circulating levels of gonadotropins and prolactin provide important information, the receptor contents in the ovary for these hormones are sometimes crucial for examining the functional state of the ovary. When mated female rats are treated with pharmacologically large doses of LH-releasing hormone (LHRH) or its agonists during preimplantation period, ovum implantation is delayed (Yoshinaga and Fujino, 1979). The delay was found to be due to reduction in the secretion of both estrogen and progesterone. This ovarian dysfunction is not due to reduction in gonadotropin secretion because the level of LH is higher than the control normal pregnant rats and FSH levels do not differ. However, LH/hCG receptor content in the ovary is found to be markedly reduced. Therefore, it is not necessarily true that the higher a hormone level in circulation, the more is the target tissue stimulated. Although the above condition is produced by the pharmacological doses of decapeptide hormones, loss of receptors or unavailability of hormone receptors may be important in the endocrine studies on ovum implantation. Translocation of estrogen-receptor complex from the cytoplasm to the nucleus is observed when estrogen exerts its action on the uterus. In view of the increasing evidence that blastocysts and the uterus can metabolize steroids and that blastocyst estrogen may be playing a role in implantation (Dickmann and Dey, 1974; Dickmann *et al.*, 1976) close examination of steroid actions on the uterus at the site of implantation appears to be important in order to elucidate the endocrine mechanisms involved in implantation. The sequential changes in the uterine sensitivity to blastocyst implantation may well be related to the uterine steroid receptor changes.

### Mice and Mongolian gerbils

Steroid hormone requirement for implantation in the mouse, and in the Mongolian gerbils appears to be similar to that in the rat, *i.e.*, both progesterone and estrogen are

necessary (Yoshinaga and Adams, 1966; Norris and Adams, 1971). Endocrine control of implantation and delayed implantation in the mouse and the rat was reviewed by Gidley-Baird (1981). Ovarian steroid secretion is necessary for implantation till the afternoon of day 4 when implantation initiates in the mouse (McLaren 1971). Since implantation takes place in the evening of day 5 in the rat, there is approximately one day difference in the time of implantation between these two species of animals. Pre-implantation estrogen secretion occurs 82 h post coitum (Finn, 1965). Bindon and Lamond (1969) hypophysectomized mated mice at different time intervals from the time of mating and found that implantation was completely prevented when hypophysectomy was performed at 2300 h on day 3 (day 1 = sperm positive day), but implantation took place in the mice whose pituitaries were removed at 0700 h on day 4. Therefore the gonadotropin secretion necessary for implantation is completed before the early morning of day 4. The pattern of preimplantation gonadotropin secretion is not clear. Bindon (1971) suggested that a combination of LH and FSH are needed for preimplantation increase in estrogen secretion. Induction of implantation in hypophysectomized mice was achieved by combinations of FSH and LH, FSH and prolactin, prolactin and LH (Gidley-Baird and Emmens, 1978). Since combination of progesterone and estrogen or progesterone and FSH, but not progesterone and LH, induced implantation in hypophysectomized mice, Gidley-Baird (1981) thinks that FSH is necessary for estrogen secretion.

### Hamsters and guinea pigs

Implantation of blastocysts can be induced with progesterone alone in ovariectomized golden hamsters (Orsini and Meyer, 1962). Therefore estrogen does not seem to be necessary for implantation in these species. However when estrogen level is measured during pregnancy, it increases prior to ovum implantation (Joshi and Labsetwar, 1972). Significance of the preimplantation rise of estrogen in the hamster is not clear. Estrogen synthetic capacity of hamster blastocysts has been suggested. Implantation in the hamster begins on the afternoon of day 5 (day 1 = sperm positive day). Implantation is not associated with any sudden increase in plasma or luteal progesterone (Lukaszewska and Greenwald, 1970). Prolactin, FSH and a trace of LH are required for the maintenance of luteal function in the hamster (Greenwald and Rothchild, 1968).

In the guinea pig very small amounts of progesterone are required for implantation, and estrogen is not necessary (Deanesly, 1960). In the guinea pig the corpora lutea require gonadotropin(s) for the first 3–4 days after ovulation and become autonomous thereafter (Aldred *et al.*, 1961). It is tempting to entertain the possibility that the blastocysts of hamster and guinea pig may produce estrogen necessary for implantation. If it is the case, the capacity of estrogen production of these tissues may be greater than that of the tissues in the rat and mouse. Aromatase activity of the uterine tissue must also be examined.

### Rabbits

Progesterone alone has been reported to induce implantation and maintain pregnancy in rabbits ovariectomized on day 5 post coitum (Pincus and Werthessen, 1938).

However, in the rabbits ovariectomized at an earlier stage of pregnancy, estrogen is also necessary for ovum implantation (Chambon, 1949).

In hypophysectomized rabbits either LH or estradiol can maintain luteal integrity and progesterone secretion. LH, however, requires the presence of follicles in the ovary, which indicates that LH stimulates estradiol production which, in turn, promotes progesterone secretion (Keyes and Nalbandov, 1976; Keyes and Armstrong, 1968; Eaton and Hilliard, 1971). Therefore estradiol is "the" luteotropin in the rabbit.

### **Pigs and sheep**

Control of luteal function in these species of animals was reviewed by Nalbandov (1973). The small laboratory animals such as rats, mice and hamsters are incomplete cyclers and the true luteal phase is lacking, thus they have short estrous cycles. Unlike these small laboratory animals, pigs and sheep are complete cyclers and the corpus luteum formed at ovulation secretes progesterone for a period of two or three weeks. In the pig the corpus luteum of the cycle is autonomous and does not require gonadotropin for formation or function. In the sheep, on the other hand, gonadotropins, particularly LH, are needed for maintenance of luteal function (Kaltenbach *et al.*, 1968; Karsch, 1970).

In the pig early embryonic attachment to the uterus and development were maintained by progesterone alone after ovariectomy on day 15, but embryonic mortality was relatively high when examined on day 25. Supplemental treatment with combinations of progesterone and estrogen reduced the high embryonic mortality (Day *et al.*, 1959). This hormonal requirement appears to be similar to that of the rabbit. Pig blastocysts have been known to have steroidogenic capacity. Perry *et al.* (1973) found that day 16 pig embryos could synthesize estrogens, but day 10 embryos could not. It has been suggested that the rise in the level of maternal plasma estrogen sulphate derived from embryos, may be a requirement for initiating blastocyst implantation locally in the uterus. In the sheep and the pig, macromolecules (proteins) produced by blastocysts prevent luteolysis at the end of the cycle (Rowson and Moor, 1967; Longnecker and Day, 1972). Read Dr. Roberts' article in this issue for more detailed account on this very interesting area.

### **Wild animals with delayed implantation**

Refer to Embryonic Diapause in Mammals (1981) for detailed account. It has been known that implantation is delayed in representatives of marsupial and eutherian mammals (Renfree and Calaby, 1981). Most mustelids (western spotted skunk, badgers, marten, wolverine etc.) always exhibit a prolonged period of delayed implantation lasting several months. In these animals luteal cells do not develop fully during the period of delayed implantation and activation of luteal cells occurs a few days before implantation. Attempts to induce implantation by injecting progesterone are not successful, suggesting that other ovarian hormone(s) are necessary for implantation (Mead, 1981). Since, the time of implantation can be hastened or delayed by changing photoperiod, neuroendocrine mechanisms are involved. Increasing the photoperiod

initiates the implantation process in the mink. Administration of a dopamine antagonist, pimozide, resulted in a rapid increase in circulating progesterone and precocious implantation (Murphy, 1983). It has been reported that prolactin increases progesterone secretion in intact mink (Papke *et al.*, 1980, Martinet *et al.*, 1981) and hypophysectomized mink (Murphy *et al.*, 1981), pimozide appears to stimulate prolactin secretion which, in turn, activates progesterone secretion. According to Mead (1981) prolactin may be important in regulating luteal function in mink and ferret but may be less significant in other mustelids such as spotted skunk and European badger. Embryonic diapause in the macropodid marsupials is also associated with undeveloped corpus luteum. Prolactin, in these species, inhibits hyperplasia and hypertrophy of luteal cells. As the prolactin level declines the corpus luteum increases in size, plasma progesterone levels rise and blastocyst resumes its development (Tyndale-Biscoe and Hinds, 1981). Thus the mechanisms involved in the embryonic diapause and resumption of the embryonic growth at the end of the diapause are variable among these species.

### Primates

Ovum implantation takes place on the 8th-9th day after fertilization in the rhesus monkey (Wislocki and Streeter, 1938; Atkinson *et al.*, 1975; Enders and Hendrick, 1980); after day 8 in the marmoset (Hearn, 1980), on the 9th-10th day in the chimpanzee (Reyes *et al.*, 1975), and on the 5½-6th day in the human (Landesman and Saxena, 1976; Ortiz and Croxatto, 1979). In the rhesus monkey, Meyer *et al.* (1969) showed that progesterone treatment of monkeys ovariectomized between the 2nd and 6th day after ovulation was able to induce implantation and maintain pregnancy. Hodgen (1983) transferred preimplantation embryos to the uterus of ovariectomized rhesus monkeys treated with estrogen followed by an estrogen-progesterone combination and obtained implantation of the transferred embryo. It remains to be determined whether estrogen has additional beneficial effect in inducing implantation in this species. This point will be discussed by Dr. Moudgal (1984) in detail elsewhere in this issue.

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## Uterine sensitivity for blastocyst implantation

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**Abstract.** Attempting to analyse the role of the ovarian hormones upon the onset, magnitude and loss of uterine receptivity/sensitivity, particular emphasis is given to uterine vascular changes. Information concerning the modulation by hormones of uterine micro-circulation appears essential for the understanding of the receptivity/sensitivity uterine changes. The generation, storage and release of vasoactive mediators and prostaglandins appear involved. As shown in the rat recently, the onset of uterine receptivity/sensitivity is temporarily correlated with the appearance of endometrial PGE binding sites under hormonal control. On the other hand catecholamines may also modulate the uterine vascular functions. Endometrial monoamine oxidase and catechol *O*-methyltransferase two enzymatic activities involved in catecholamine deactivation show hormone dependant changes parallel to the manifestation of uterine receptivity/sensitivity. The precise role of these phenomena is discussed.

**Keywords.** Uterine; receptivity/sensitivity; blastocyst.

Irrespective of the highly variable way in which egg-implantation occurs in different species, all the events related to this process appear to follow a well-timed schedule. Thus, around the time at which the fertilized egg reaches the blastocyst stage, the uterus enters a phase of optimal conditions for co-participation.

We present here several criteria characterizing this uterine phase of receptivity for blastocyst implantation as defined mainly in rats. Among them we will more particularly describe the endometrial vascular reactivity towards the blastocyst stimulus or towards artificial stimuli leading to a decidual response. This reactivity is expressed by an increase in capillary permeability and appears to be, in all the species studied, a regular index of endometrial receptivity/sensitivity. It may be the result of a cascade-like effect involving various vasoactive tissular mediators, such as histamine and/or certain prostaglandins, just as a common inflammatory reaction.

The existence of an optimal time for the application of the stimulus which will initiate a decidual response is known since the classical studies of Loeb (1908). It has been further shown by De Feo (1967) that under controlled light conditions, the uteri of pseudopregnant rats acquire sensitivity to a knife-scratch trauma between days 5 and 6 of pseudopregnancy. By using the intraluminal injection of chemical inducers instead of a knife-scratch, De Feo demonstrated further that a peak of sensitivity appears around noon of day 5.

Histological examination of rat uteri fixed around noon of day 5 of pregnancy or pseudopregnancy shows an oedematous swelling of the endometrial stroma (Psychoyos, 1967). This transient stromal oedema is one of the phenomena which have to be considered as indicating the manifestation of optimal uterine conditions for

nidation. It occurs at the mid-luteal period, independently of the presence of an implanting ovum, in a variety of species including the human (Noyes *et al.*, 1950). The obliteration of the uterine cavity resulting from this oedema must be essential for the implantation process, facilitating the primary contact of the blastocyst with the epithelial surface. Closure of the uterine lumen may also result from some specific functions of the luminal epithelium, namely pinocytosis and endocytosis. These epithelial activities must also be considered as indicators of the sensitive/receptive period. They must contribute to the removal of fluid from the uterine lumen but also to the uptake of material released eventually by the blastocyst.

In the rat, an ultrastructural correlate of the receptive endometrium is the presence, on the luminal surface of the epithelial cells, of sponge-like structures which can be observed under scanning electron microscopy (Psychoyos and Mandon, 1971). They appear to be "pinopodes" involved in the pinocytosis and endocytosis phenomena (Enders and Nelson, 1973; Parr and Parr, 1974; Parr, 1980). Abundant on the day of optimal sensitivity, they disappear thereafter. The presence of similar structures has also been observed in the human endometrium. According to our own observations, they are clearly distinguishable on days 18 and 19 of the cycle, are completely developed on days 20 and 21 and have largely regressed by days 22 and 23 of the cycle (Martel *et al.*, 1981). Their presence in the human endometrium appears therefore limited to 24–48 h around the perinidatory period, indicating that the period of optimal conditions for egg-implantation must be short in our species too.

In the rat, either the natural decidualization due to blastocyst stimulus or the experimentally obtained decidual reaction is preceded by a dramatic increase in the permeability of the endometrial capillaries (Psychoyos, 1967). This vascular reactivity appears to be a *sine qua non* condition for the decidual response of the endometrium. Any procedure inhibiting this increase in permeability also inhibits decidualization. In a parallel way, the only period during which the endometrium is able to exhibit a change in vascular permeability in response to a deciduogenic stimulus, is also the only period during which such an endometrial stimulation leads to decidualization. The degree of increase in capillary permeability, observed for instance after a traumatic stimulation, parallels the endometrial sensitivity for a post-trauma decidual reaction (Psychoyos and Casimiri, 1980).

Similar observations, concerning the occurrence of an increased capillary permeability at the initiation step of the egg-implantation process, have been made in all the species studied. However, the factors linked to this phenomenon are not as yet defined with certainty. In the pathway leading to endometrial capillary reactivity, we can presume that a primary step may be the production of histamine through an activation of histidine decarboxylase, located near or in the endothelium of uterine small vessels. As known, histamine has been proposed by Shelesnyak several years ago to be involved in decidualization (Shelesnyak, 1957). Recent findings have contributed to renew interest in this vasoactive amine. As shown in the rabbit by Dey and his collaborators, the intraluminal injection of a specific inhibitor of histidine decarboxylase on day 5 of pregnancy, interrupts the implantation process (Dey *et al.*, 1978). Furthermore, in this species, the blastocyst itself exhibits a significant histidine decarboxylase activity which appears to reflect its own capacity to produce histamine (Dey *et al.*, 1979a). In addition, rabbit endometrial cells were found to contain  $H^1$  receptors (Dey *et al.*, 1979b) via

which, in other systems, histamine binding can mediate vasodilation and increase vascular permeability.

It is considered that in general, during an inflammatory process, the vascular leakage induced by histamine is potentiated by prostaglandin generation. In the rat, in areas of increased vascular permeability induced by blastocysts or artificial stimuli, the concentration of prostaglandins of the *E* and *F* series and of prostacyclin are found to be increased (Kennedy and Zamecnick, 1978). On the other hand, the inhibitor of prostaglandin synthesis, indomethacin, given to rats on day 5 of pregnancy, inhibits the increase in endometrial vascular permeability in a transitory way (Kennedy, 1977). In pseudopregnant rats treated with this inhibitor, the intra-uterine injection of a saline/gelatin mixture does not increase the endometrial vascular permeability unless it contains prostaglandin  $E^2$  (Kennedy, 1979).

Kennedy *et al.* (1983 a,b) examined recently the hypothesis that uterine receptivity for blastocyst implantation and sensitivity for decidualization are controlled by the presence of receptors for prostaglandin  $E^2$  in the endometrium. Our studies performed in rats led to the first demonstration of specific binding of prostaglandin  $E^2$  to the endometrium of any species. A substantial increase in the concentration of endometrial binding sites for PGE is observed between days 4 and 5 of pseudopregnancy (Kennedy *et al.*, 1983a). In ovariectomized animals the presence of binding sites for this prostaglandin was found to be progesterone dependant and detectable only when progesterone had been administered for 48 h or more. It is also of interest to know that the binding sites for PGE<sup>2</sup> were found to be located only in the stroma (Kennedy *et al.*, 1983b). The role of the luminal epithelium may be in this case to produce this prostaglandin or to allow the transit of PGE produced by the blastocyst although both alternatives are also possible.

The decidual metamorphosis of the endometrium requires the involvement of the epithelium. Injury of the endometrial stroma without disruption of the integrity of the luminal epithelium does not induce decidualization (Fainstat, 1963). Similarly, the ability of traumatic stimuli to induce a decidual response is abolished if the luminal surface of the uterus is exposed to  $-20^{\circ}\text{C}$  for a few minutes (Ferrando and Nalbandov, 1968) or if the luminal epithelium is removed mechanically *in vivo* (Lejeune *et al.*, 1981). According to our recent observations, ablation of the epithelium interferes also with the occurrence of a post traumatic increase in capillary permeability (unpublished data). Obviously some factor essential for the uterine vascular reactivity is missing under these conditions.

Catecholamines may also modulate the vascular reactivity of the uterus. Monoamine oxidase and catechol *O*-methyl transferase, the enzyme complexes involved in their deactivation, show an increased activity during the luteal phase. We have shown that in the rat, the endometrial activity of both of these enzymes increases sharply by day 3 of pseudopregnancy remaining from then on at a plateau of high level (Rath *et al.*, 1979).

Catechol *O*-methyl transferase is also involved in catechol estrogens metabolism. The catechol structure of these hydroxylated estrogen products appears to confer to them an increased chemical reactivity and their physiologic role is the subject at the present time of intensive studies. Catechol estrogens appear to be involved in prostaglandin production. In homogenates of rat uteri, addition of 2-hydroxyestradiol increases the production of certain prostaglandins 4 fold, as compared with the increase induced by



the addition of estradiol-17 $\beta$  (Kelly and Abel, 1980). Through their agonistic or antagonistic effects towards estrogens, the catechol estrogens may also participate in the hormonal control of vascular reactivity of the uterus, particularly through the bias of their effects on prostaglandin production.

It is still too early for conclusions concerning the chain of events involved in uterine receptivity for nidation. It can however be considered that in terms of vascular reactivity, the presence of binding sites for prostaglandins at the stromal level favours their involvement in this reactivity. It seems also that the luminal epithelium is an indispensable intermediate for the vascular reactivity preceding decidualization. There are in these new informations several points which deserve to be further explored.

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## Epithelial cell function during blastocyst implantation

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**Abstract.** In response to the ovarian secretion of progesterone and estrogen during early pregnancy, the mammalian uterus develops the capacity to perform complex cellular activities which occur before and after blastocyst implantation. Luminal epithelial cells participate in regulation of the metabolism of the blastocyst through the control of its humoral environment, provide an appropriate matrix for changes to occur at the interface between trophoblast and epithelium, and appear to transmit information from the blastocyst to the underlying stroma to initiate decidualization. With the completion of these functions during implantation in rodents, the epithelial cells self-destruct and are removed by phagocytic activity of the trophoblast. Control of both the endocytotic and secretory activity of luminal epithelial cells and their eventual self-destruction would require regulation of the Golgi-endoplasmic reticulum-lysosomes system within these cells. Progesterone secretion during early pseudopregnancy increases levels of cathepsin D, a lysosomal proteinase, in luminal epithelial cells by increasing the rate of enzyme synthesis. Progesterone pretreatment of ovariectomized rats followed by estradiol treatment results in the development of uterine sensitivity to decidualogenic stimuli. The number of proteins which are synthesized by luminal epithelial cells in response to estradiol to achieve this sensitivity has been determined. Epithelial cytosol proteins from rats treated with medroxyprogesterone acetate (3.5 mg sc) or medroxyprogesterone acetate plus estradiol (200 ng sc) were separated by two dimensional polyacrylamide gel electrophoresis. The synthesis of two proteins increased after 8 h of estradiol treatment and the synthesis of another three was increased by 12 h. The increased synthesis of these proteins could be related to changes in the capacity of the luminal epithelial cell for prostaglandin synthesis. The epithelial capacity for prostaglandin synthesis increases during pseudopregnancy to maximum levels at the time of maximum sensitivity to decidualogenic stimuli. Epithelial prostaglandin synthetic capacity may also depend upon the accumulation of prostaglandin precursors within these cells. Estradiol treatment of medroxyprogesterone acetate pretreated ovariectomized rats increased the arachidonic acid content and composition of epithelial phosphatidyl choline but the increases were not statistically significant. These changes in protein and lipid synthesis controlled by progesterone and estrogen would appear to contribute to the cellular activities of the luminal epithelium during early pregnancy.

**Keywords.** Cell function; blastocyst; implantation.

During early pregnancy, epithelial cells lining the uterine lumen perform several cellular functions vital to blastocyst implantation. By secretion and endocytosis, epithelial cells control the uterine environment of the blastocyst. Changes in the composition of the epithelial apical membrane enable the adhesion and attachment of the blastocyst. Arrival of the blastocyst initiates the transmission of a message, which increases capillary permeability and initiates the decidualization of underlying stromal cells. The accumulation of lysosomal hydrolase activity within the luminal epithelial

Abbreviations used: GERL, Golgi-endoplasmic reticulum-lysosomes; MPA, medroxyprogesterone acetate; IMP, intramembraneous particles; PGF<sub>2α</sub>, prostaglandin F<sub>2α</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; E, estradiol; sc, subcutaneous; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

cells could provide enzymatic capacity for several of these functions and for the eventual self-destruction of the epithelial cells which occurs during blastocyst implantation in some species. This brief review will not consider all of these topics equally but will tend to focus on more recent data obtained in rodents.

From species to species there is considerable diversity in the length of time that the blastocyst remains free within the uterus and in the cellular response of the luminal epithelium to the blastocyst (Enders and Given, 1977). The amount and kinds of protein secreted by the luminal epithelium are presumably related to these kinds of diversity. Protein and other components of uterine fluid provide nutrition for the free blastocyst, and a means of control of blastocyst metabolism and development. Lumina of uterine glands are dilated by the progressive accumulation of carbohydrate material during the periimplantation period indicating increasing secretory activity (Given and Enders, 1980, 1981). Numerous studies have examined the composition of uterine fluid and its hormonal control to determine the mechanisms by which blastocyst development and uterine sensitivity are synchronized. These studies have been recently reviewed (Surani and Fishel, 1980) and the topic will be considered in more detail by other contributors to this symposium. The suggestion that delayed implantation in rodents results from the secretion of inhibitors of blastocyst metabolism and that activation following estradiol results from a loss of activity of the inhibitors continues to be actively investigated (McLaren, 1973; Psychoyos, 1973; Weitlauf, 1976, 1978; O'Neill and Quinn, 1983).

In addition to secretion, epithelial cells can control the environment of the blastocyst by endocytotic uptake of material from the uterine lumen. The morphology of two endocytotic pathways which are active during the period of uterine sensitivity have been described (Parr, 1980, 1982a,b). Pinopod vacuoles and pinocytotic vesicles formed at the apical surface of luminal epithelial cells are channelled into secondary lysosomes. In the second pathway, pinocytotic vesicles formed at the basal and lateral membranes move across the cell towards the luminal surface, where they may fuse with the apical membrane to release their contents into the uterine lumen by exocytosis. Vesicles derived from the Golgi complex, also appear to move toward the apex of the cell and fuse with the surface membrane.

The endocytotic and secretory activity of uterine luminal epithelial cells requires intense intracellular activity by the Golgi-endoplasmic reticulum-lysosomes (GERL) system. Material taken into the cell by endocytosis is sequestered and digested within lysosomes (Parr, 1982a). Proteins for secretion synthesized on ribosomes on the rough endoplasmic reticulum are inserted through the membrane into the microsomal lumen. During this insertion, proteolytic cleavage and glycolytic processing of the protein determine whether the protein will be secreted or sequestered within the lysosomes of the cell. Histochemical studies have identified concentrations of lysosomal enzymes in endometrial epithelial cells which are controlled by progesterone and estrogen (Woessner, 1969; Roy *et al.*, 1983; Moulton and Koenig, 1983, Sengupta *et al.*, 1979). During early pregnancy, both multivesicular bodies and more typical lysosomes are found in luminal and glandular epithelial cells (Enders and Given, 1977).

Our studies have demonstrated that cathepsin D, a lysosomal proteinase, is concentrated in luminal and glandular epithelial cells of the uterine endometrium (figure 1). During early pseudopregnancy, the levels of cathepsin D activity and the rate



**Figure 1.** Rat uterine cross section through non-implantation site on day 6 of pregnancy. Uterus was fixed in Bouin's solution, dehydrated, and embedded in paraffin. Deparaffinized sections ( $6\mu\text{m}$ ) were pretreated with methanolic  $\text{H}_2\text{O}_2$ , and then with goat anti-rat serum to cathepsin D, donkey anti-goat IgG, and goat peroxidase anti-peroxidase. Sections were reacted with diaminobenzidine and  $\text{H}_2\text{O}_2$ , washed, dehydrated, and mounted.

of synthesis of this enzyme increase to maximum levels at the time of maximum uterine sensitivity to decidualogenic stimuli (Moulton and Koenig, 1983). Considerable evidence implicates the lysosome as an important site of intracellular protein degradation (Segal and Doyle, 1978). Accumulation of cathepsin D within the epithelium could contribute to the degradation of extracellular protein brought to lysosomes by endocytosis and to the eventual programmed cell death of the luminal epithelium which is a significant feature of blastocyst implantation in rodents.

Adhesion and attachment of the blastocyst to luminal epithelial cells during early pregnancy could depend upon the insertion or deletion of specific proteins from the apical membrane of the epithelium. Although this potential mechanism of uterine sensitization has not been thoroughly examined, there is considerable morphological change in surface structures of luminal epithelium during the periimplantation period (Nilsson, 1980; Enders *et al.*, 1980; Takahashi *et al.*, 1980). Prior to implantation, the surface of the luminal epithelium is covered by regular digitiform microvilli. Later the microvilli are flattened and bulbous cytoplasmic projections appear for a limited period of time. With the loss of uterine sensitivity, these projections disappear and the epithelial cells bear ordered microvilli once again. These changes are largely under the control of the maternal ovarian hormones. Pronounced morphological differences between the microvilli of the implantation chamber and those of the rest of the uterus

have been observed, but these differences could not be related to differences in the chemical properties of the surface coat of the uterus (Enders *et al.*, 1980). The glycocalyx on the surface of rat uterine luminal epithelial cells contains both acidic and neutral carbohydrates under several different hormonal conditions. The acidic carbohydrates appear to be influenced by hormones, but no evidence could be found for a similar influence of hormones on neutral carbohydrates as revealed by concanavalin A binding (Murphy and Rogers, 1981). Further study may identify other sugars or specific acceptor proteins in the apical membrane of the epithelium. After separating labelled uterine epithelial proteins by 2 dimensional gel electrophoresis, we can detect the increased synthesis of several proteins and the altered proteolytic processing or glycosylation of several others during uterine sensitization to deciduogenic stimuli (figure 2). However, the location of these proteins within the luminal epithelial cells and their identity has not been determined.

More recently, freeze fracture electron microscopy has been used to examine more fine structure of the apical membrane and the number and density of intramembranous particles (IMP's), in particular (Murphy *et al.*, 1982a). IMP's are thought to represent globular proteins, or proteins complexed with small amounts of lipid and embedded deeply in the hydrophobic interior of the lipid bilayer. Aggregations of IMP's within the membranes may have some significance in promoting cell to cell contacts and several studies have implicated aggregations of IMP's in membrane transport. During early pregnancy, the density of IMP's in the apical membrane of luminal epithelial cells increase and by day 6, complex arrays of the IMP's were observed. If aggregates of IMP's participate directly in blastocyst implantation, then increase in the fluidity of the apical membrane to enhance the formation of these aggregates could be a significant prerequisite of implantation (Murphy *et al.*, 1982b). Increased membrane fluidity could also enable the aggregation of epithelial receptors for histamine, prostaglandins, or estradiol which have been proposed as mediators of communication between the blastocyst and the uterus. Receptors for prostaglandins (Kennedy *et al.*, 1983) have been measured in the endometrium, but their presence in epithelial cells has not been demonstrated. Progesterone pretreatment necessary for the development of uterine sensitivity to deciduogenic stimuli does not appear to increase estrogen binding in luminal epithelial cells (Martel and Psychoyos, 1982; Quarmby and Martin, 1982).

For many cells, the methylation of membrane phospholipid appears to be an initial common pathway for the transduction of receptor mediated signals through the membranes (Hirata and Axelrod, 1980). Various amines and peptides interacting with cell surfaces initiate a cascade of biochemical and physical changes in membranes including increased phospholipid methylation, increased mobility of receptors because of decreased membrane viscosity, the generation of cAMP, histamine release, mitogenesis, and chemotaxis. In several cell types examined, phospholipid methylation was associated with calcium influx, the release of arachidonic acid, and the formation of lysophosphatidylcholine. Contact of the blastocyst with the cell membrane of luminal epithelial cells initiates histamine release, the generation of cAMP, increased calcium influx, and increased prostaglandin synthesis.

In recent studies, we have examined changes in the activity of phosphatidyl methyltransferase activity in luminal epithelial cells following hormonal treatment to induce uterine sensitization to deciduogenic stimuli. Ovariectomized rats were treated

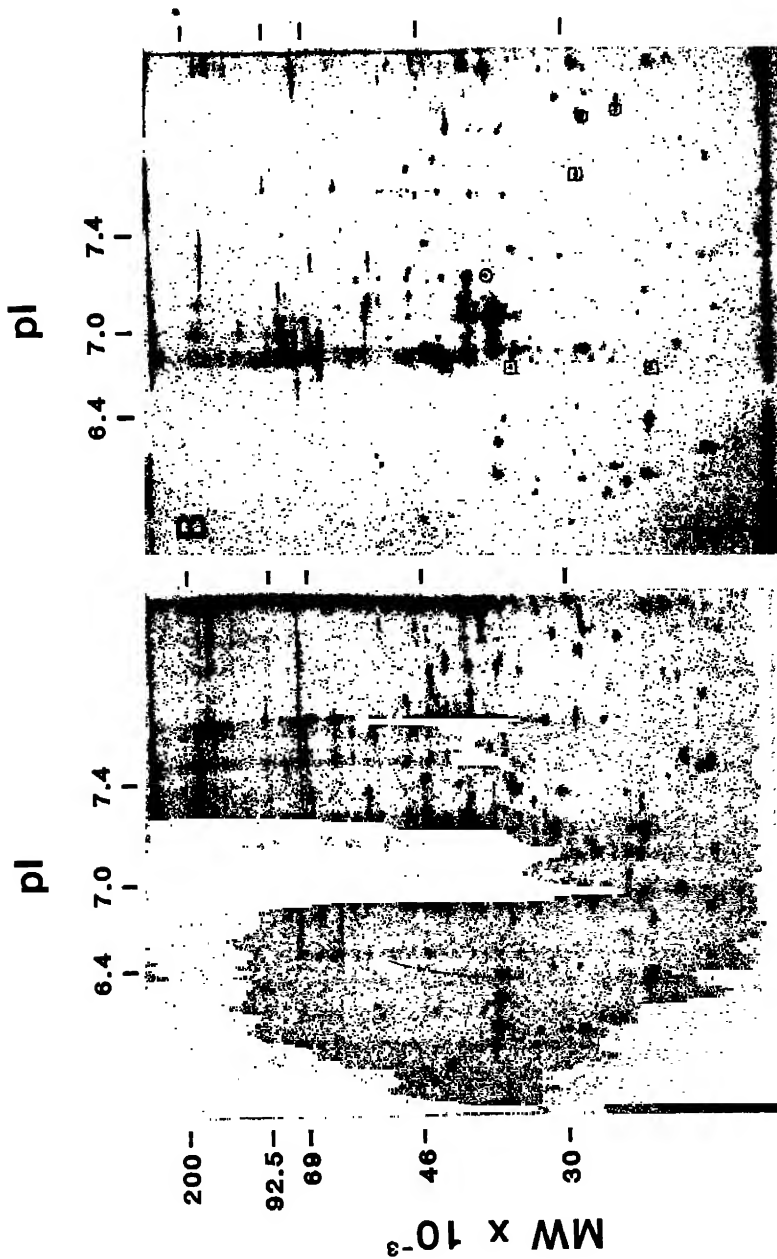


Figure 2. Effect of estradiol on protein synthesis in the uterine luminal epithelium of rats pretreated with progesterone. On successive days ovariectomized rats were treated with E, E, 0, MPA, 0, and vehicle or estradiol for 12 h (E, estradiol, 500 ng, sc; 0, no treatment; MPA, medroxyprogesterone acetate, 3.5 mg, sc; e, estradiol, 200 ng, sc). Uterine horns were incubated in phosphate-buffered saline containing [ $^{35}$ S]-methionine (400  $\mu$ Ci/ml) for 1 h. Epithelial cell extracts were prepared as described by Garrels (1979), and then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and isoelectric focusing electrophoresis.

with progestin (medroxyprogesterone acetate, 3.5 mg) for 48 h before treatment with estrogen (estradiol, 200 ng). Estradiol treatment had no effect on levels of either phosphatidyl-methyltransferase I or II activity in uterine luminal epithelial cells. Although increases in the activity of these enzyme activities do not appear to be prerequisite for the development of uterine sensitivity, these preliminary results do not preclude the involvement of epithelial membrane lipid methylation during the earliest uterine response to the blastocyst.

With the recognition of the presence of the blastocyst, epithelial cells appear to transmit a message to underlying stromal cells to initiate decidualization and increase capillary permeability (Lundkvist, 1978). Stromal decidualization and edema precede deterioration and degeneration of the luminal epithelium by some 12–24 h (Finn, 1977; Finn and Porter, 1975). When the luminal epithelium is experimentally removed from uterine horns sensitized by pretreatment with progesterone and estrogen, the uteri become insensitive to both traumatic and non-traumatic stimuli (Lejeune *et al.*, 1981). The inability of de-epithelialized horns to decidualize was not overcome by intraluminal injections of epithelial tissue homogenate, prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) or prostaglandin  $E_2$  ( $PGE_2$ ), arachidonic acid or histamine. Detachment of the epithelium without removal also prevented decidualization. However, after the epithelium had regenerated, most of decidual cell development was restored. These studies indicate that the luminal epithelium is an obligatory transducer of a message from the luminal surface of the cell to the underlying stromal cells (Lejeune *et al.*, 1981). Luminal cells at the site of blastocyst implantation appear to synthesize and release deciduogenic substance(s) or to initiate changes in membrane permeability in response to these substances.

Increases in endometrial vascular permeability initiated by the blastocyst may involve both histamine and the prostaglandins, but considerable research implicates the prostaglandins as deciduogenic substances (Kennedy and Armstrong, 1981). Concentrations of prostaglandins are elevated in areas of increased vascular permeability after deciduogenic stimuli, and inhibition of prostaglandin synthesis by indomethacin delays or inhibits increases in vascular permeability and the decidualization of stromal cells (Kennedy and Armstrong, 1981). Concentrations of  $PGE$ ,  $PGF$ , and 6-keto- $F_{1\alpha}$  are significantly elevated in implantation sites relative to non-implantation regions of the uterus. The production of  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  by uterine homogenates peaked on day 5 of pregnancy, the day of implantation (Fenwick *et al.*, 1977; Phillips and Poyser, 1981). To determine the histological site of this production, we prepared epithelial cell samples during early pseudopregnancy with and without indomethacin. When epithelial cells were prepared in the presence of indomethacin, lower tissue levels of prostaglandins were observed and these values did not change during pseudopregnancy (figure 3). When epithelial cells were prepared in the absence of indomethacin, levels of PG's were 4 to 5 times greater and the greatest increase occurred on day 5 of pseudopregnancy.

Changes in prostaglandin synthetic capacity could result from changes in the activity of enzymes necessary for synthesis or from changes in levels of lipid precursor for prostaglandin synthesis. During early pregnancy and pseudopregnancy, the content, composition, and distribution of lipid stores in luminal epithelial cells respond to progesterone and estrogen secretion (Enders and Given, 1977; Beall, 1972). Specific

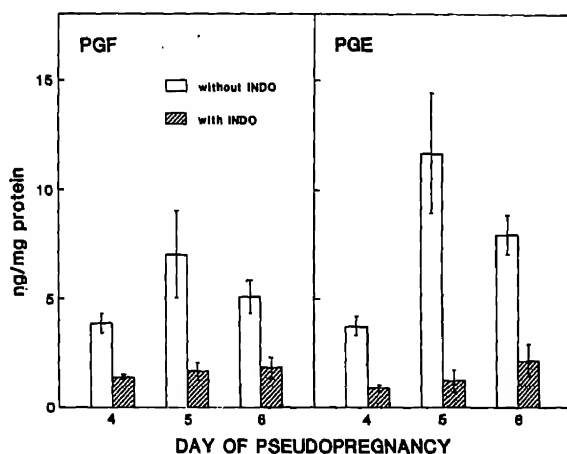


Figure 3. Levels of prostaglandins in uterine luminal epithelial cells during pseudopregnancy. Epithelial cells were removed, homogenized with or without indomethacin (20  $\mu$ g/ml), and centrifuged (6000  $g$ , 20 min). Prostaglandin levels in the supernatants were determined by radioimmunoassay using PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> as standards. Each value is the mean  $\pm$  the SEM of at least 7 samples with 3 rats/sample.

histochemical techniques demonstrated that triacylglycerol, the predominant neutral lipid in intracellular lipid droplets of uterine epithelial cells, decreased during early pregnancy (Boshier *et al.*, 1981). Further decreases in stores of neutral lipid were observed in implantation sites (Boshier, 1976). Neutral and unsaturated lipids decreased in response to estradiol as phospholipid granules appeared in the apical cell cytoplasm of luminal epithelial cells (Gould *et al.*, 1978). Biochemical assays of homogenates of uterine horns demonstrated that progesterone increased total uterine lipid content by increasing levels of phospholipid and neutral lipid (Manimekalai *et al.*, 1979). Estrogen treatment resulted in decreases in total uterine lipid content, but increased levels of phospholipids (Manimekalai *et al.*, 1979; Sakai *et al.*, 1980). The development of endometrial sensitivity to deciduogenic stimuli could depend upon these effects of progestin and estrogen on uterine lipid.

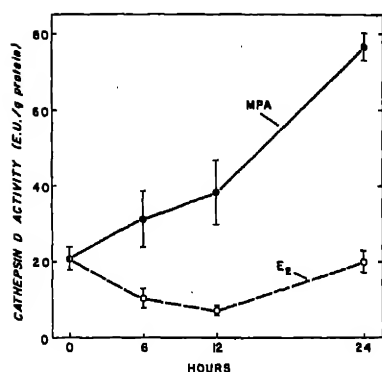
Rats ovariectomized for 3 weeks were pretreated with estradiol and progestin to develop uterine sensitivity to a deciduogenic stimulus. On each successive day, rats received the following treatments: E, E, 0, 0, MPA, 0, e or vehicle (E, estradiol, 500 ng, sc; 0, no treatment; MPA, medroxyprogesterone acetate, 3.5 mg, sc; e, estradiol, 200 ng, sc). After 18 h, control and e treated animals were sacrificed, phospholipids were extracted from the luminal epithelial cells, and separated by two dimensional tlc (Portoukalian *et al.*, 1978; Skipski *et al.*, 1964). The fatty acid composition of each phospholipid was determined by gas chromatography (MacGee and Allen, 1974). The precursor of prostaglandin synthesis, arachidonic acid, was detected only in phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). Estradiol increased the level of arachidonic acid in PC and the per cent of composition but neither result was statistically significant ( $p < 0.1$ , nil). These data do not support the hypothesis that uterine sensitivity to deciduogenic stimuli or the capacity for prostaglandin synthesis depend upon changes in uterine lipid content or composition.



With the completion of various cellular functions prior and during blastocyst implantation, the epithelial cells self-destruct and are removed by phagocytic activity of the trophoblast (Schlafke and Enders, 1975). Although the trophoblast initiates cellular death of luminal epithelial cells, these cells appear programmed by progesterone and estrogen secretion to self-destruct given an appropriate stimulus (Finn, 1977; Finn and Publicover, 1981). Both the destruction of luminal epithelial cells during implantation in several rodent species and the digestion of endocytosed material depend upon the intracellular activity of lysosomes and their content of hydrolytic enzymes (El-Shershaby and Hinchliffe, 1975; Parr and Parr, 1978). In several examples of epithelial cell death, early lysosomal participation takes the form of first autophagy and then autolysis as acid hydrolases leak out of the autophagic vacuoles and damage the cytoplasm (Wyllie, 1981). It is not clear whether a lysosomal mechanism initiates cell death, but lysosomes are clearly involved in mammary gland involution (Helminen and Ericsson, 1970), prostate regression (Helminen and Ericsson, 1971), and blastocyst implantation (El-Shershaby and Hinchliffe, 1975; Abraham *et al.*, 1970; Elangovan and Moulton, 1980a; Moulton and Ingle, 1981).

In our early experiments, we measured the activities of several lysosomal enzymes in blastocyst implantation sites and in non-implantation uterine tissue. Of the five lysosomal enzymes examined, only cathepsin D activity decreased following implantation (Moulton, 1974; Moulton *et al.*, 1978). Immunohistochemical localization was used to determine whether the change in cathepsin D activity involved a specific change in one lysosomal enzyme in all uterine cells or a change in the enzyme content of a specific cell type. Cathepsin D was concentrated in luminal and glandular epithelial cells, and the enzyme content of luminal epithelial cells decreased as these cells deteriorated in response to either the blastocyst or an artificial stimulus (Elangovan and Moulton, 1980a; Moulton and Ingle, 1981). Lysosomal activity and cathepsin D activity have been shown to increase prior to cell death associated with anuran metamorphosis (Lockshin, 1981), involution of the mammary gland (Helminen and Ericsson, 1970), and prostate regression (Helminen and Ericsson, 1971). The concentration of cathepsin D in uterine luminal epithelial cells suggested that epithelial cell death during blastocyst implantation might depend in part upon the accumulation of this lysosomal protease.

Using a method for removing uterine luminal epithelial cells (Fagg *et al.*, 1979), we examined changes in the activity and rates of synthesis of cathepsin D in these cells during early pseudopregnancy (Moulton and Koenig, 1983). The activity of epithelial cathepsin D increased during pseudopregnancy and attained maximal levels as the uterus developed sensitivity to decidualogenic stimuli. The rate of epithelial cathepsin D synthesis increased dramatically between days 2 and 3 of pseudopregnancy and remained elevated. The activity and rates of synthesis of cathepsin D in stromal-myometrial tissues, however, remained unchanged during early pseudopregnancy. To determine the hormonal control of the rate of epithelial cathepsin D synthesis, ovariectomized rats were treated with MPA progesterone or estradiol (figure 4). The progestins increased the activity and rates of synthesis of cathepsin D in luminal epithelial cells. Estradiol had little effect on the relative rate of synthesis of cathepsin D and did not enhance the uterine response to progestins (Elangovan and Moulton, 1980b; Moulton, 1982). Estradiol treatment following progestin pretreatment, however, was necessary for the decrease in cathepsin D activity in response to a



**Figure 4.** Effect of MPA or estradiol ( $E_2$ ) on cathepsin D activity in uterine luminal epithelial cells. Ovariectomized rats were injected with MPA (2 mg, sc) or estradiol (5  $\mu$ g, ip) and 15 animals were sacrificed at each interval thereafter. Each point represents the mean  $\pm$  SEM of at least 6 assays. Each assay determined the cathepsin D activity of 5 pooled uterine horns.

deciduogenic stimulus (Moulton, 1982). The effect of estradiol could be inhibited by compounds which stabilize lysosomal structure. Collectively, these studies suggested that progesterone and estrogen might control autophagic activity and cell death in luminal epithelial cells during implantation by control of both lysosomal enzyme content and intracellular activity.

Despite the diversity of the morphology of implantation among species, cells lining the uterine lumen in all species must perform similar and essential functions during early pregnancy. While the timing and appearance of these cellular functions may vary, the expectation persists that biochemical processes held in common may still be found. Elucidation of these biochemical processes in luminal epithelial cells and their hormonal control continues to have considerable merit.

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## Physiological aspects of blastocyst uterine interaction

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**Abstract.** An interaction between the blastocyst and the uterus is essential for establishment of pregnancy. Because maternal estrogen is not an absolute requirement, estrogen of embryonic origin has been implicated in this process in the pig and the rabbit. Furthermore, estrogen forming capacity has been documented in the blastocyst of these species. However, while the complete machinery for steroid synthesis in the pig blastocyst has been demonstrated, the issue is still unresolved for the rabbit blastocyst. In the present communication we have shown that  $17\alpha$ -hydroxylase and C17-20-lyase, enzymes involved in the formation of androgens ( $C^{19}$ -steroids) from  $C^{21}$ -steroids (progestins), are present in day-6 rabbit blastocysts. C17-20-lyase activity was undetectable to low in day-5 and increased in day-6 blastocysts. The activity was further increased in day-6 blastocysts cultured for 24 h. Because prostaglandins have been implicated in uterine vascular changes at about the time of implantation and pregnancy establishment, and because catechol estrogens are more potent than phenolic estrogens in stimulating prostaglandin synthesis in the blastocyst and the uterus, we determined catechol estrogen forming capacity in the rabbit and pig blastocyst. Catechol estrogen forming capacity (estrogen-2/4-hydroxylase) in the pig blastocyst appears on day 10 of pregnancy, peaks on day 12 and then declines. Our preliminary experiments also indicate that day-6 rabbit blastocysts have catechol estrogen forming capacity. On the basis of our present findings and of others, we propose that catechol estrogens of embryonic origin mediate the stimulatory effect of estrogens on prostaglandin synthesis in the embryo and/or the uterus and thus participate in the process of establishment of pregnancy.

**Keywords.** Blastocysts; implantation; pregnancy recognition; catecholestrogens; prostaglandins; aromatase.

## Introduction

Corner (1947) wrote, "... the uterine chamber is actually a less favorable place for early embryos than, say, the anterior chamber of the eye, except when the hormones of the ovary act upon it and change it to a place of superior efficiency for its new functions". More recent is the realization that the establishment of pregnancy results from the culmination of an intimate interaction between the developing embryo and the differentiating uterus. However, although conceptually accepted, the nature and timing

Abbreviations used: PGs, Prostaglandins; P<sub>3</sub>, pregnenolone; 2-OH-E<sub>2</sub>, 2-hydroxyestradiol; 4-OH-E<sub>2</sub>, 4-hydroxyestradiol.

of such a two-way interaction between the blastocyst and the uterus are still a challenging question. The establishment of early pregnancy consists of several synchronized and precisely controlled embryonic and maternal components: (a) the migration, spacing and orientation of the embryo into the uterus, (b) apposition and adhesion of the trophoblast with the uterine epithelium followed by attachment to the uterus (the process of implantation), (c) increased capillary permeability and blood flow in the uterine vascular bed, and (d) prolongation of ovarian luteal function for maintenance of progesterone secretion.

Although these events present intricate problems in endocrine control and involve the participation of the embryo, the endocrine capabilities of the conceptus have not been fully appreciated in the past. This issue deserves special attention in the case of certain animals in which the establishment of early pregnancy including implantation can occur in the absence of maternal estrogen. Pigs, sheep, rabbits and hamsters fall into this category. Two possibilities have been considered: (i) either estrogen is not required for pregnancy establishment in these species, or (ii) if required, this is provided by the blastocyst. Recently, both estrogen and prostaglandins (PGs) have been implicated in this process (George and Wilson, 1978; Heap *et al.*, 1981; Hoversland *et al.*, 1982; Pakrasi and Dey, 1982; Davis *et al.*, 1983). The synthesis of estrogen by the pig blastocyst was first reported in 1973 (Perry *et al.*, 1973) and subsequent experiments have confirmed this finding (Heap *et al.*, 1981). Although the question of estrogen formation in the rabbit blastocyst has been a subject of great debate, the estrogen forming capacity in the blastocyst of this species has recently been documented independently by three different laboratories (George and Wilson, 1978; Hoversland *et al.*, 1982; Wu and Lin, 1982). However, while the complete machinery for steroid synthesis in the pig blastocyst has been established (Heap *et al.*, 1981), the issue is still unresolved for the rabbit blastocyst. Also unanswered is the physiological significance of embryonic estrogen in the control of early pregnancy. Recently, it has been observed that catechol estrogens, the major metabolites of phenolic estrogens, are more potent than the latter in stimulating PG synthesis in the embryo and the uterus *in vitro* (Kelly and Abel, 1980, 1981; Pakrasi and Dey, 1983). In order to find answers to the above questions, we have investigated whether the rabbit-blastocyst has the capacity to convert progestins to androgens (17 $\alpha$ -hydroxylase and C17-20-lyase activities) and whether the pig and rabbit blastocysts have catechol estrogen forming capacity. On the basis of our findings and of others we will discuss the possible role of embryonic catechol estrogens in the establishment of early pregnancy through generation of PGs in the blastocyst and/or the uterus. Rabbits and pigs were chosen because of the availability of a relatively large amount of embryonic tissues with which to work.

## Materials and methods

### *Preparation of tissues*

**Rabbit:** New Zealand white rabbits were induced to superovulate (Mukherjee *et al.*, 1978) and blastocysts were recovered on days 5 and 6 of pregnancy (day 1 = 24 h *post coitum*). In addition, day-6 blastocysts were cultured for 24 h in RPM1-1640 medium supplemented with crystalline bovine serum albumin (Sigma Chem. Co., St. Louis,

Missouri, USA) under an atmosphere of 90% N<sub>2</sub>, 5% CO<sub>2</sub> and 5% O<sub>2</sub> at 37°C in order to obtain embryonic age approximately equivalent to day-7 of pregnancy. At the end of incubation, the blastocysts were washed in 0.1 M HEPES buffer (pH 7.4) containing NADPH and stored at -80°C until assayed. After thawing, blastocysts were homogenized in assay buffer using a micro-ultrasonic cell disruptor with a 2.5 mm tip (Kontes, Evanston, Illinois, USA). The homogenate was centrifuged at 105,000 g for 60 min at 4°C. The pellet was resuspended in assay mixture for determining 17 $\alpha$ -hydroxylase and C17-20-lyase activities.

**Pig:** Yorkshire and Yorkshire  $\times$  Duroc gilts were checked once daily for estrus and mated on the first and second days of estrus. Blastocysts were recovered surgically from the uterus on days 10-14 of pregnancy (Davis and Day, 1978; Davis *et al.*, 1983). The onset of estrus was considered as day 0 of pregnancy. Blastocysts were examined under a microscope, and those with apparently normal morphology were placed in 12  $\times$  75 glass tubes and kept frozen at -80°C until assayed for estrogen 2-/4-hydroxylase.

#### Assay procedures

**17 $\alpha$ -hydroxylase:** The assay is based on the liberation of [<sup>3</sup>H]-OH from 17 $\alpha$ -[<sup>3</sup>H]-pregnenolone (P<sub>3</sub>) as a result of 17 $\alpha$ -hydroxylase activity (Kremers, 1976; Tsai-Morris and Johnson, 1982). The [<sup>3</sup>H]-OH liberated enzymatically was distilled under vacuum and counted in a liquid scintillation counter. The reaction mixture in a final volume of 200  $\mu$ l contained 100  $\mu$ M 17 $\alpha$ -[<sup>3</sup>H]-pregnenolone (1.34 mCi/mmol, prepared and characterized by Dr. P. Kremers, University of Liege), an NADPH generating system (Hoversland *et al.*, 1982) and blastocyst tissue extract. The incubation was carried out in 16  $\times$  75 mm polycarbonate tubes for 2 h at 37°C in a Dubnoff metabolic water bath. At the end of the incubations, the steroids in each sample were extracted with 2 ml of diethyl ether. The aqueous phase was distilled to determine the amount of pregnenolone consumed, while the ether phase was subjected to Sephadex L-20 column chromatography to separate 17-OH-pregnenolone from pregnenolone. The fraction with 17-OH-pregnenolone was measured by radioimmunoassay (Grotjan and Johnson, 1974; Johnson, 1979). Enzyme activity was calculated, after subtracting blank values obtained with boiled tissue extracts or without tissue extracts.

**C17-20, lyase:** The assay is based on the liberation of [C<sup>14</sup>]-acetic acid from 21-[<sup>14</sup>C]-progesterone as a result of C17-20-lyase. The assay method used in determining the lyase activity in the blastocyst has been adopted from Chasalow *et al.* (1982) and modified by Johnson and Griswold (1983). Although 17-OH-progesterone has been the substrate of choice in the past, the above groups of investigators have shown that progesterone is the preferred substrate for this enzyme.

The reaction mixture in a final volume of 200-300  $\mu$ l in 0.1 M HEPES buffer (pH 7.3) contained 40  $\mu$ M 21-[<sup>14</sup>C]-progesterone (48.9 mCi/mmol, New England Nuclear Corp., Boston, Massachusetts, USA), an NADPH generating system (Johnson and Griswold, 1983) and blastocyst tissue extracts. The incubation was carried out in 16  $\times$  75 mm polycarbonate tubes at 37°C for 1-2 h. The reaction was stopped by the addition of 400  $\mu$ l of 0.01 N HCl. The tubes were centrifuged at 2300 g for 15 min, the supernatant fluid was distilled under reduced pressure and the distillate containing



[C<sup>14</sup>]-acetic acid was counted in a liquid scintillation spectrometer with a 91% efficiency for [C<sup>14</sup>]. The radioactivity in the blank samples, lacking only the tissue extracts, was subtracted from the experimental values. The enzyme activity was expressed as microgram of substrate consumed per embryo per hour.

#### *Estrogen 2-/4-hydroxylase*

The assay is based on isolation of 2-hydroxyestradiol (2-OH-E<sub>2</sub>) and 4-hydroxyestradiol (4-OH-E<sub>2</sub>) by a two-step separation procedure involving the use of neutral alumina column followed by thin layer chromatography as developed by Hersey *et al.* (1981). Blastocysts were sonicated in 1–2 volumes of 0.3 M sucrose. Duplicate samples and blanks were incubated for 10 min at 30°C in a final volume of 150  $\mu$ l 0.1 M HEPES buffer (pH 7.4) containing 10  $\mu$ M [6-7-<sup>3</sup>H]-estradiol (40–60 Ci/mmol, New England Nuclear Corp.), 10  $\mu$ M ascorbic acid and 3.3  $\mu$ M 2-OH-E<sub>2</sub>. The reaction was terminated by the addition of cold buffer containing [4-<sup>14</sup>C]-2-OH-E<sub>2</sub> which was used to correct for losses of both 2- and 4-hydroxyestradiol. Tritiated 2-OH-E<sub>2</sub> and 4-OH-E<sub>2</sub> were isolated by adsorption to neutral alumina and separated from each other by silica gel thin layer chromatography. Activity was expressed as pmol/mg protein/10 min. Blank values were obtained by measuring activity of tissues heated for 10 min at 100°C.

## Results

#### *17 $\alpha$ -hydroxylase activity in the rabbit blastocyst*

The activity of 17 $\alpha$ -hydroxylase (pg substrate consumed/blastocyst/2 h) in day-6 blastocysts cultured for 24 h was  $45.10 \pm 6.8$  as determined from the liberation of [<sup>3</sup>H]-OH (4 pools of 879 blastocysts, 120–280 blastocyst in each pool). The assay of 17-OH-pregnenolone by radioimmunoassay, following column chromatography, was about 13% of pregnenolone (without correction for the recovery losses) that was hydroxylated. It is possible that pregnenolone was metabolized to androgens *via* 17 $\alpha$ -hydroxylase-C17-20-lyase complex or alternatively, pregnenolone was converted to progesterone *via*  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase (EC1.1.1.145). We also measured 17 $\alpha$ -hydroxylase activity in the 100,000 g pellet of one batch of 170 day-6 blastocysts; the activity was 50.48 pg substrate consumed per blastocyst per hour.

#### *C17-20-lyase activity in the rabbit blastocyst*

As shown in table 1, lyase activity was undetectable to low on day-5 and increased on day-6. The activity was further increased in day-6 blastocysts cultured for 24 h. It should be noted that a considerable variation in enzyme activities was observed between batches of blastocysts of the same embryonic age. This variation in enzyme activity could result from storage of blastocysts for several weeks in order to collect sufficient numbers, a great variation in the sizes of the blastocysts or from interassay variations. Indeed, in three occasions, we have failed to detect any lyase activity in comparable number of day-6 blastocysts. Nonetheless the enzyme activity was

Table 1. C17-20-lyase activity in rabbit blastocysts.

No. of expts.	No. of blastocysts/ expt.	Age of embryos (days)	activity (pg substrate consumed/embryo/h)
1	126	5	2.35*
6	90-187	6	10.85 $\pm$ 4.09
4	25-116	6**	37.43 $\pm$ 15.90

Values are mean  $\pm$  S.E.M.

\* Activity was undetected in another experiment with 72 blastocysts.

\*\* Day 6 blastocysts cultured for 24 h.

detectable in most of the cases and we are confident that lyase activity is present in the preimplantation rabbit blastocysts.

#### *Estrogen 2-/4-hydroxylase activity in the pig blastocyst*

As shown in table 2, total estrogen 2-/4-hydroxylase was detectable on day-10 of pregnancy, peaked on day-12 and then declined. In this experiment, 4-hydroxylation ranged from 15 to 25% of 2-hydroxylation. The activity was concentrated in the extra-embryonic portion of the blastocyst and is similar to that observed for aromatase activity (table 3).

Table 2. Estrogen 2-/4-hydroxylase activity in pig blastocyst.

Age of embryos	No. of expts	Estrogen 2-/4-hydroxylase (pmol/mg protein/10 min)	
		2-OH-E <sub>2</sub>	4-OH-E <sub>2</sub>
10	3	0.38 $\pm$ 0.38	0.27 $\pm$ 0.14
11	4	14.08 $\pm$ 8.85	3.38 $\pm$ 2.03
12	7	66.06 $\pm$ 12.94	11.62 $\pm$ 2.12
13	3	45.07 $\pm$ 22.89	7.50 $\pm$ 3.99
14	3	7.40 $\pm$ 5.39	1.04 $\pm$ 0.74

Values are mean  $\pm$  S.E.M.

5-9 blastocysts from a single pig were used in each experiment. The increase in activity between days 10 and 12 and the decrease between days 12 and 14 were statistically significant ( $P < 0.05$ ).

## Discussion

Although an indication of conversion of [C<sup>21</sup>]-steroids to [C<sup>19</sup>]-steroids (androgens) by the preimplantation rabbit blastocyst was obtained (Huff and Eik-Nes, 1966), no conclusive studies regarding the activities of 17 $\alpha$ -hydroxylase and C17-20-lyase required for conversion of [C<sup>21</sup>]-steroids to androgens have been reported. Because of the participation of these enzymes in the formation of androgens which then can be

**Table 3.** Aromatase and estrogen 2-/4-hydroxylase activities in embryonic and extra-embryonic tissues from pig blastocysts.

	Aromatase (pmol/mg protein/h)	Estrogen 2-/4-hydroxylase (pmol/mg protein/10 min)	
		2-OH-E <sub>2</sub>	4-OH-E <sub>2</sub>
Extraembryonic	60.60 ± 25.14	30.20 ± 9.90	8.20 ± 4.70
Embryonic	13.10 ± 7.27 (n = 2)	ND (n = 4)	ND (n = 4)

Aromatase activity was determined by measuring the amount of <sup>3</sup>H<sub>2</sub>O formed during incubation with [1 $\beta$ -<sup>3</sup>H]-androstenedione (Weisz *et al.*, 1982). Measurement of estrogen-2-/4-hydroxylase was performed as described in methods and material. Enzyme activities were measured in an equal number of embryonic and extraembryonic tissue samples obtained from the same blastocysts (2-9 blastocysts per sample). Activity is expressed as mean  $\pm$  S.E.M., obtained from the number of samples indicated (n).

ND - Not detectable; less than two times blank activity.

aromatized to estrogens, the study of these enzymes deserved special attention, especially in the light of the present findings of aromatase activity in the rabbit blastocyst (George and Wilson, 1978; Hoversland *et al.*, 1982; Wu and Lin, 1982). The results of our present investigation demonstrate that both 17 $\alpha$ -hydroxylase and C17-20-lyase are present in the rabbit blastocyst. It should also be noted that the C17-20-lyase activity in the blastocyst of this species can be detected by using progesterone as the substrate. Recent investigations (Chasalow *et al.*, 1982; Johnson and Griswold, 1983) on rat testis and ovary have also shown that progesterone is the preferred substrate over 17-hydroxyprogesterone for C17-20-lyase activity. This raises the possibility that the formation of 17-hydroxyprogesterone may not be an obligatory step in the formation of androstenedione from progesterone. Further investigation, however, will be required to answer this problem.

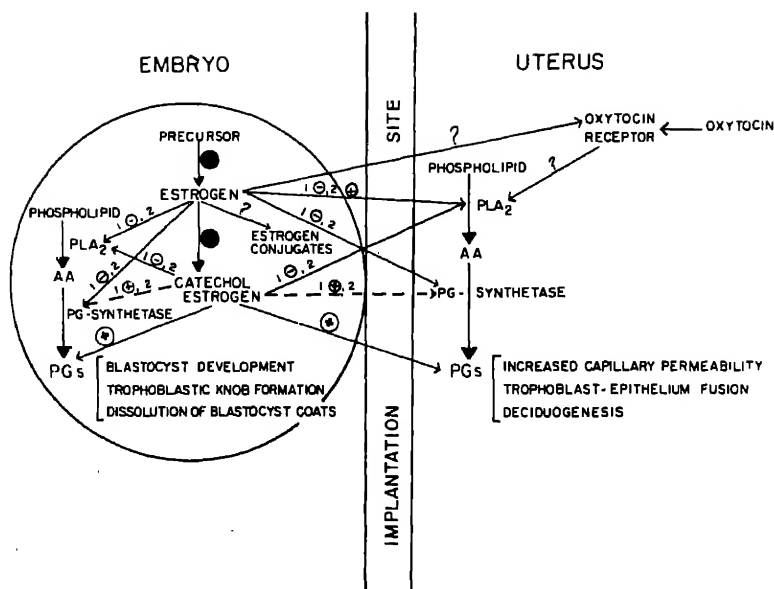
The failure of other investigators to show androgen or estrogen forming capacity in the rabbit blastocyst could be due to the use of an inadequate number of blastocysts in the reaction mixture, an unsaturating concentration of the substrate, the lack of addition of optimal concentration of co-factors, or the technique used. In most of the studies in the past, conventional techniques of product isolation by chromatography were used and very little care was taken to prevent further metabolism of the products to be identified. A failure to use saturating concentrations of substrates may be true for several steroidogenic studies with rabbit blastocysts. Furthermore, the use of a large number of blastocysts appears to be required in order to obtain measurable amounts of steroid production.

Although it appears from this present and earlier investigations that the rabbit blastocyst has the capacity to form estrogen from progesterone *in vitro*, we do not know whether they synthesize androgens or estrogens *in vivo*. On the other hand, the evidence for formation of estrogen in the pig blastocyst both *in vivo* and *in vitro* has been documented (Heap *et al.*, 1981). The next question is: if both rabbit and pig blastocysts are capable of synthesizing estrogens, what is the fate and physiological significance of

this embryonic estrogen? PGs are considered to participate in the process of pregnancy establishment including implantation and estrogen is a known modulator of PG synthesis in the uterus *in vivo* (Ham *et al.*, 1975; Pakrasi *et al.*, 1983). Despite the popular agreement on the receptor mediated uterotrophic action of estrogen, the mechanism by which this steroid stimulates PG synthesis in the uterus is not clearly understood. The studies of Castracane and Jordan (1976), where stimulation of PG production in the rat uterus by estrogen was unaffected by inhibitors of protein and RNA synthesis or an antiestrogen, support a non-genomic function. Furthermore, the inability of estrogen to stimulate PG production in the uterus *in vitro* (Pakrasi and Dey, 1983), in contrast to its effects *in vivo*, suggests that the metabolism of estrogen *in vivo* may be involved in PG synthesis. Indeed, recent reports indicate that catechol estrogens, the major metabolites of phenolic estrogens, are more potent than the latter in stimulating PG synthesis in the uterus and blastocyst (Kelly and Abel, 1980, 1981; Pakrasi and Dey, 1983). Therefore, our findings of catechol estrogen forming capacity in the pig blastocyst appears interesting. We have also preliminary evidence for catechol estrogen forming capacity in the rabbit blastocyst. The timing of peak catechol estrogen forming capacity in day-12 pig blastocyst coincides with the appearance of aromatase activity (Heap *et al.*, 1981) and of PGs in the embryo (Davis *et al.*, 1983) as well as with changes in uterine blood flow (Ford and Christenson, 1979). The surge of estrogen-2-/4-hydroxylase (catechol estrogen forming capacity) in the pig blastocyst appears at a critical time *i.e.* just after the completion of the process of migration, spacing and orientation of the blastocyst in the uterus (Dhindsa *et al.*, 1967; Heuser and Streeter, 1929) and just before the adhesion of the trophoblast to the uterine epithelium and its definitive attachment on day 18 of pregnancy (Crombie, 1972). Because changes in uterine blood flow requires the presence of the embryo, the PGs that appear in both the blastocyst and in uterine flushings at this time are thought to be responsible for uterine vascular changes (Davis *et al.*, 1983).

The idea that catechol estrogens formed by the embryo act at or close to their site of synthesis, is compatible with the recent view regarding function of this class of estrogen metabolites (Merriam and Lipsett, 1983). The rapid clearance of catechol estrogens from the circulation makes it unlikely that they function as circulating hormones (Merriam *et al.*, 1980). It is more likely that catechol estrogens serve as a local or paracrine function acting on the cells in which they are formed or on those in their close proximity. Estrogen, the substrate for estrogen-2-/4-hydroxylase, should be available locally since aromatase activity has been identified in the preimplantation blastocysts of pig and rabbit. Moreover, both aromatase and estrogen-2-/4-hydroxylase are concentrated in the same extra-embryonic portion of the pig blastocyst. These various findings lead us to propose that the stimulation of PG synthesis responsible for the vascular changes in the uterus and attributed to phenolic estrogens, may in fact be mediated by their catechol metabolites (figure 1).

Stimulation of PG synthesis is only one of several mechanisms through which catechol estrogen may mediate their function. Like the phenolic estrogens, catechol estrogens can act through the receptor (Hersey *et al.*, 1982). The addition of a second hydroxyl group to the A-ring of estrogens, however, reduces the affinity of these steroids for the cytosolic estrogen receptor (Martucci and Fishman, 1976). Therefore, catechol estrogen formation could serve as a means to reduce the potency of the parent



**Figure 1.** A proposed mode of action of catechol estrogens in stimulating prostaglandin synthesis in relation to the embryo-uterine interaction during early pregnancy. This model has been proposed on the basis of our work in the rabbit. However, this model now also appears to be applicable to the pig.

AA: Arachidonic acid, PLA<sub>2</sub>: phospholipase A<sub>2</sub>; 1: Direct effect; 2: Receptor mediated effect?; (---) Proposed effect; (⊖), No known effect; (⊕), Evidence for stimulatory effects has been obtained for the indicated event; (●), Evidence has been obtained for the indicated pathway.

estrogen within the target cell. Another intriguing set of potential mechanisms of action of catechol estrogens could be mediated *via* catechol structure of these metabolites. As catechols, they are potent inhibitors of tyrosine hydroxylase and catechol-*O*-methyltransferase, enzymes involved in the synthesis and degradation of catecholamines, respectively (Lloyd and Weisz, 1978; Ball *et al.*, 1976). However, there are no data at present to suggest how these mechanisms may be relevant to the establishment of early pregnancy.

Although catechol estrogen formation by the blastocyst is proposed to work *via* stimulation of PG synthesis in the embryo and/or the uterus and thereby causing vascular changes in the uterus that precede implantation or attachment of the blastocyst with the uterine epithelium, the role of catechol estrogen mediated stimulation of PG synthesis in the maintenance of functional corpus luteum especially in the pig cannot be excluded.

In summary, we have shown that the rabbit blastocyst has the capacity to form estrogen from progesterone and both the pig as well as the rabbit blastocysts have the potential to convert phenolic estrogens to catechol estrogens. The effects of catechol

estrogens are likely to include modulation of PG synthesis but further work will be required to establish this hypothesis.

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## Metabolism in preimplantation mouse embryos

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**Abstract.** The ability of preimplantation mouse embryos to utilize glucose oxidatively is controlled, in part at least, at the level of glycolysis. Various experimental observations are reviewed that indicate the regulatory mechanism in delayed implanting blastocysts involves the classic negative allosteric feedback of high levels of ATP on phosphofructokinase while the situation in 2-cell embryos appears to be more complicated. That is, in addition to the usual negative effect of ATP and citrate on phosphofructokinase, there appears to be a modification of hexokinase that prevents phosphorylation of adequate amounts of glucose and results in low levels of fructose-6-phosphate at the 2-cell stage and consequently there is a failure to release the inhibition of phosphofructokinase even if ATP and citrate levels decrease. Although both types of embryos have limited glycolytic activity, they do have adequate capacity for citric acid cycle activity and oxidative phosphorylation, and are able to maintain a high ATP:ADP. It is argued, therefore, that the reduced levels of macromolecular synthesis characteristic of 2-cell and delayed implanting blastocysts are not due to restricted energy substrates or regulatory controls on glycolysis and a subsequent low energy state. On the contrary, it seems that the reduction in oxidative utilization of glucose in these situations is a result of diminished energy demand because of the low level of synthetic activity. The potential significance of this relationship between energy production and utilization in terms of potential regulatory mechanisms in preimplantation embryos is discussed.

**Keywords.** Preimplantation; mouse; embryos; carbohydrate metabolism; delayed implantation.

### Introduction

Interest in the capacity of mammalian preimplantation embryos to utilize various energy substrates was initiated by Whitten's (1956, 1957) observations that mouse embryos at the 8-cell stage would develop in a Krebs-Ringer bicarbonate solution containing albumin and glucose while 2-cell embryos required the addition of lactate. This demonstration of a difference in substrate requirements led to the realization that there are changes in the metabolic capacity of mammalian preimplantation embryos with development, and suggested that some regulatory mechanism must function even in these very early stages. Although a great deal of information about the metabolic capabilities of early mammalian embryos has been generated in the 25 years since Whitten's original observations, and some control mechanisms have been elucidated (Biggers, 1971; Wales, 1973, 1975), the significance of changes in substrate utilization is still not entirely clear. The purpose of this communication is to review some of the work that has been done with normal and delayed implanting embryos in an attempt to identify questions that remain unanswered.



### Cleavage stage embryos

Almost ten years after Whitten's original papers, Brinster (1965) reported the results of a systematic study undertaken to evaluate the effects of a wide variety of potential energy substrates on development of 2-cell mouse embryos *in vitro*. He found that development would occur if the embryos were supplied with lactate, pyruvate, oxaloacetate, or phosphoenolpyruvate, but not if they were given acetate, citrate,  $\alpha$ -ketoglutarate, succinate, fumarate, malate, or glucose. In further studies by Brinster and his colleagues utilization of  $O_2$  was measured (Mills and Brinster, 1967), and it was demonstrated that lactate and pyruvate could be oxidized equally as well by 2-cell and 8-cell embryos (Brinster, 1967a) while Quinn and Wales (1973a,b) reported that these same substrates would maintain normal levels of ATP in both 2-cell and 8-cell embryos. These findings have been taken as indicating that enzyme systems necessary for citric acid cycle activity and oxidative phosphorylation are intact and functional even in 2-cell embryos. The assumption then, has been that glucose and those citric acid cycle intermediates that do not support development of the early embryos, either cannot enter the cells or cannot be converted to a substrate that will support continued operation of the citric acid cycle.

Embryos develop the capacity to use other substrates between the 2-cell and the 8-cell stages (Brinster and Thomson, 1966) and most attention in this regard has been directed at malate and glucose. Wales and Biggers (1968) were unable to demonstrate either accumulation of labelled carbon or evidence of oxidative utilization of U- $[^{14}C]$ -malate by 2-cell embryos in contrast to 8-cell embryos and concluded that absence of a transport mechanism for malate was, in the early stage, responsible. It should be noted however, that initial rates of uptake of malate were not determined in those studies and therefore, that changes in permeability were not measured directly. Another argument for there being a change in the capacity of embryos to take up malate has been developed from data generated in several different experiments. First, it will be recalled that 2-cell embryos develop *in vitro* with oxaloacetate but not malate as the sole source of energy supplied to the medium (Brinster, 1965); and, second, that 2-cell embryos have  $NAD^+$  dependent malate dehydrogenase activity (Brinster, 1966) and thus should be able to convert malate to oxaloacetate. Although it seems from this that malate should support early embryos if it could enter the cells, one further point must be considered; oxaloacetate cannot be used directly to support continued operation of the citric acid cycle, rather it must be converted to acetyl-coenzyme A presumably through the formation of pyruvate. Although this does not appear to have always been appreciated (Biggers *et al.*, 1967), it raises the question of whether the conversion occurs intracellularly and if so, by means of which enzymatic pathway and in which cellular compartment? There are several possibilities (figure 1): Oxaloacetate might enter the cells and be converted to pyruvate in the cytoplasm; this would presumably require formation of phosphoenolpyruvate (*i.e.*, *via* phosphoenolpyruvate carboxykinase) and then pyruvate (*i.e.*, *via* pyruvate kinase); this scheme seems reasonable although neither of the enzymes necessary for these transformations have been demonstrated at early stages of development. It is also possible that oxaloacetate is reduced to malate in the cytoplasm (malate dehydrogenase activity has been demonstrated at the 2-cell stage; Brinster, 1966; Quinn and Wales, 1971) and then goes on to pyruvate (*i.e.*, also in the

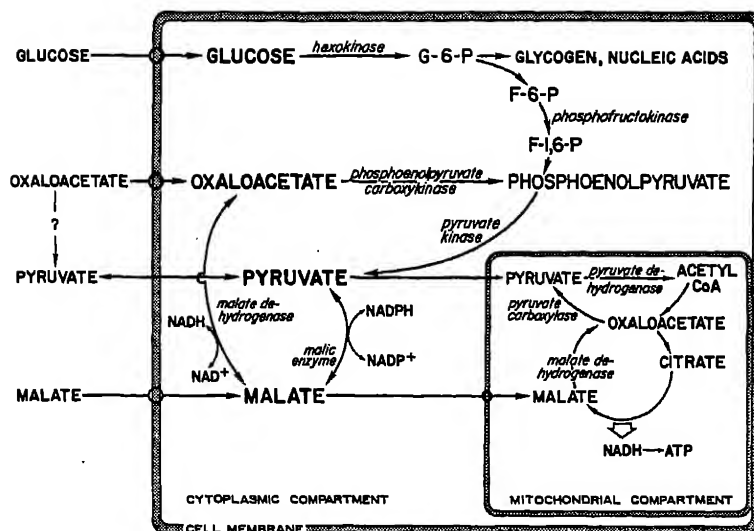


Figure 1. Possible pathways for carbohydrate metabolism in preimplantation embryos.

cytoplasm *via* malic enzyme; the presence of malic enzyme has been demonstrated (Quinn and Wales, 1971). Alternatively, malate from the cytoplasm might enter the mitochondria (malate, but not oxaloacetate, can cross the mitochondrial membrane), be converted back to oxaloacetate, and then on to pyruvate (*i.e.*, by way of the mitochondrial enzyme pyruvate carboxylase). Enzyme activities necessary for these mitochondrial conversions have been demonstrated in early mouse embryos (Quinn and Wales, 1971). Although this scheme might seem unlikely because it would eventually lead to a deficiency of reducing equivalents in the cytoplasm, it is compatible with Thomson's (1967) finding that malonate (an inhibitor of pyruvate carboxylase) will block development of 2-cell embryos supplied with oxaloacetate but not those given pyruvate. Thomson's (1967) observation also argues against the possibility that oxaloacetate is able to support development only because it spontaneously breaks down to pyruvate in the medium. Thus, although it is not clear how oxaloacetate is utilized, it has generally been assumed that it supports the citric acid cycle either directly (Biggers *et al.*, 1967), or after being converted intracellularly to pyruvate by way of one or another of the schemes outlined above. From this assumption it has been argued that there are changes in transport of malate with development. The reasoning is as follows: since the enzymatic capability to convert malate to oxaloacetate is present at the 2-cell stage, the inability of malate to support development at that stage indicates it is not able to enter embryos. That malate is capable of supporting 8-cell embryos then, indicates that capacity for its transport develops at that stage. It must be emphasized that this does not constitute a rigorous proof of a change in permeability, nor has the possibility that malate can enter 2-cell embryos but for some reason is not converted to oxaloacetate or pyruvate (possibly the absence of malic enzyme or a high NADH to NAD<sup>+</sup> ratio) been considered. These questions will have to be resolved before the

problem of why some citric acid cycle intermediates will support early development, and others will not, can be answered definitively.

Utilization of glucose by 2-cell embryos has been examined in detail and although it is apparently involved in synthesis of glycogen and other macromolecules (Stern and Biggers, 1968; Brinster, 1969; Murdoch and Wales, 1973; Wales, 1975), it is not oxidized to any great extent (Brinster, 1967b) and will not maintain normal levels of ATP (Quinn and Wales, 1973b). Glucose will, however, support development of 8-cell embryos (Brinster, 1965) and again it has been proposed that this is because of changes either in enzyme pathways (in this case Embden-Myerhoff scheme), or in the embryo's capability to transport the substrate. The observation by Wales and Brinster (1968) that 2-cell and 8-cell embryos incubated in 5.1 mM U- $[^{14}\text{C}]$ -glucose accumulate labelled carbon at equal rates in the first 3 min supports the suggestion that it is regulation of glycolysis rather than uptake that is responsible for the inability of glucose to support the early embryos. However, changes in glucose transport between the 2-cell and 8-cell stage have not been conclusively ruled out. More recently, the various intermediates in glycolysis have been measured in 2-cell, 8-cell, and later stage embryos during starvation and refeeding with glucose or pyruvate (Barbehenn *et al.*, 1974, 1978). Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, ATP, AMP, orthophosphate ( $P_i$ ), citrate, isocitrate, malate and  $\alpha$ -ketoglutarate were measured and it was found that there is a "block" at the phosphofructokinase step in 2-cell embryos. Although phosphofructokinase is allosterically inhibited by high levels of citrate and ATP in many systems, the block is characteristically lost under anaerobic conditions as levels of citrate and ATP fall (*i.e.*, the Pasteur effect). This does not seem to be the case in starved 2-cell embryos where citrate and ATP levels also fall, since phosphofructokinase activity remains low during refeeding with glucose. Furthermore, it appeared that the potential for adequate hexokinase activity was present in the early embryos (*i.e.*, when measured in cell lysates), but maximum levels of the hexosephosphates after refeeding were lower in the early stages than they were in the later stages. This was interpreted as indicating that for some unknown reason glucose phosphorylation is shut off in 2-cell embryos by a low level of glucose-6-phosphate and thus, only a low level of fructose-6-phosphate is attained. Since fructose-6-phosphate can potentially release the inhibition of phosphofructokinase, it was proposed that an increase in that compound at a later stage is the major cause of the greater phosphofructokinase activity and hence glycolysis. Although it was concluded that the true cause of the inability of 2-cell embryos to utilize glucose is an "unknown modification" of the initial enzymes in the glycolytic scheme (*i.e.*, hexokinase, Barbehenn *et al.*, 1978), no definitive explanation has been offered.

Studies such as these have added greatly to our understanding of metabolism in early embryos but they have not provided satisfactory answers to the original question of whether it changes in transport or enzyme pathways that are primarily responsible for differences in the ability of glucose and various citric acid cycle intermediates to support development at the 2-cell and 8-cell stages of development. Furthermore, although we know that the level of macromolecular synthesis is low in these early embryos, and that as a consequence ATP levels are relatively high, we do not have any information on the significance of putative changes in pathways for various energy intermediates to embryos that are normally in an environment that is rich in pyruvate (Nieder and Corder, 1983).

## Delayed implanting embryos

Development of mouse embryos is arrested at the blastocyst stage in mothers that are concurrently lactating (lactational delayed implantation) or are ovariectomized before noon on the fourth day of pregnancy (experimentally delayed implantation). Although it has been known for some time that metabolic and mitotic activity in these embryos decreases until the suckling young are removed or estrogen is injected into the mother (McLaren and Menke, 1971; McLaren, 1973), and many reports have appeared that deal with metabolic activity and synthesis of macromolecules, there has not really been a satisfactory explanation, at the molecular level, for the embryonic quiescence associated with delayed implantation. Typically in these experiments, embryos are recovered from the uteri of mice that have been ovariectomized and treated with progesterone for several days and incubated *in vitro* with radioactive precursor molecules. Utilization or incorporation of label into DNA, RNA, or protein is then estimated by scintillation counting or autoradiographic techniques. The findings can be summarized briefly as follows.

### DNA

Incorporation of [ $^3\text{H}$ ]-thymidine slows (and finally ceases altogether) as embryos enter the dormant phase associated with lactational delayed implantation (McLaren, 1968). This occurs in the 24–30 h following removal of the maternal ovaries in experimental delayed implantation (Given, 1983; Given and Weitlauf, 1981) and might have been anticipated as the cells become arrested in the  $G_1$  phase of the cell cycle (Sherman and Barlow, 1972); the level of mitotic activity has been shown to decrease over the same interval by direct cell counts (Weitlauf *et al.*, 1979). Furthermore, reactivation of the embryos by injecting estrogen into the mother (or simply removing them from the uterus to appropriate culture conditions), leads to a resumption of [ $^3\text{H}$ ]-thymidine incorporation (Given and Weitlauf, 1981, 1982) and cell division (Weitlauf *et al.*, 1979). Taken alone, the changes in incorporation of labelled thymidine might be interpreted as reflecting changes in permeability or specific activities of internal precursor pools. But taken in conjunction with the fact that there are corresponding changes in cell division and the observation that the cells are in  $G_1$  during the dormant period, it seems clear that the reported changes in rates of incorporation of [ $^3\text{H}$ ]-thymidine actually reflect changes in synthesis of DNA.

### RNA

Several investigators have compared the incorporation of [ $^3\text{H}$ ]-uridine by delayed implanting blastocysts with that by embryos implanting normally or undergoing the process of reactivation. Typically, blastocysts were recovered from the uteri of ovariectomized females and incubated *in vitro* with [ $^3\text{H}$ ]-uridine and it was observed that the rate of incorporation of label into RNA was lower in delayed implanting embryos than that in normal or reactivated embryos (Weitlauf, 1976; Chavez and VanBlerkom, 1979). However, because the specific activities of the endogenous pools of UTP were not known the actual rates of RNA synthesis could not be determined in those experiments and there has been disagreement over interpretation of the results. More recently a similar series of experiments was performed and the specific activities

of the endogenous pools of UTP were determined and the actual rates of synthesis of RNA could be calculated from the rates of incorporation of [ $^3\text{H}$ ]-uridine (Weitlauf and Kiessling, 1980). In this way, after correcting for differences in numbers of cells it was shown that the overall rate of synthesis of RNA in delayed implanting embryos is about one-third of that in reactivated embryos. It was also shown that the increases in synthesis of RNA that occurred with reactivation took place gradually over 12–24 h *in vitro*. A similar change in synthesis of RNA has been shown to occur *in vivo* following the injection of estrogen into the mother (Weitlauf, 1982). Furthermore, in that experiment it was shown that although the bulk of the increase in [ $^3\text{H}$ ]-uridine incorporation was into rRNA, there was a transient burst of mRNA synthesis early in the process of reactivation (*i.e.*, 3–4 h after the injection of estrogen). Although these results with RNA are reminiscent of those with DNA, it should be noted that in contrast to that situation, the embryos do not completely stop synthesis of RNA during delayed implantation. Rather, they continue to operate at approximately one-third of the level characteristic of active embryos presumably to support synthesis of 'house keeping' proteins.

### *Protein synthesis*

The rate of incorporation of labelled amino acids into protein by delayed implanting mouse embryos is about half that observed in normal implanting or reactivated embryos; it increases to normal levels within a few hours of the injection of estrogen or removal of the blastocysts from the uterus to a suitable culture medium (Weitlauf, 1974; VanBlerkom *et al.*, 1979). Although the effects of potential differences in specific activities of intracellular amino acid pools have not been rigorously evaluated, it has been shown that total protein content changes proportionately with the incorporation of radioactive amino acids (Weitlauf, 1973) and the estimates of protein synthetic rates from incorporation of label would seem to be valid. Although it is clear from such observations that the rate of protein synthesis in delayed implanting embryos is low and that it increases with reactivation, the unanswered question is whether this involves transcription of specific new mRNA or a more generalized change in metabolic activity and rates of translation. It would be expected that the mechanisms responsible for the developmental arrest associated with delayed implantation will be quite different, depending on which of these strategies has been employed by the embryos. Several relevant observations have been made but the question remains unanswered. Thus, two-dimensional electrophoresis has revealed that some 'new' proteins can be demonstrated in the first few hours of the reactivation process (VanBlerkom *et al.*, 1979); unfortunately, it is not clear whether these "spots" are truly new proteins and thus reflect synthesis of new mRNA or simply represent increases in amounts of 'old' proteins that become visible because of increased rates of synthesis. Attempts to block the process of activation *in vitro* with  $\alpha$ -amanitin (*i.e.*, at doses that would be expected to block synthesis of new mRNA) do not completely prevent the expected increases in incorporation of amino acids (unpublished results). The difficulty with this approach is, that because it is total incorporated radioactivity that is measured, one cannot determine if small changes in synthesis of specific proteins have occurred. Finally, it has been reported that the amounts of some enzymes increase, others remain the same, or

even decrease with activation (Weitlauf *et al.*, 1979; Weitlauf, 1981; Nieder and Weitlauf, 1983), again suggesting that there are some differential changes in protein synthesis. However, it must be emphasized that such changes could reflect either transcriptional or translational mechanisms. Thus, the question of how macromolecular synthesis is regulated in delayed implanting mouse embryos remains unanswered.

### Glucose utilization

In addition to the studies on macromolecular synthesis there have been numerous experiments directed to utilization of glucose as an energy substrate. The original observations were those of Menke and McLaren (1970a,b; Menke, 1972) that demonstrated the production of  $^{14}\text{CO}_2$  from U- $^{14}\text{C}$ -glucose was reduced in delayed implanting embryos. In these experiments again, blastocysts were recovered and incubated with labelled substrate and radioactive product generated with time was evaluated by scintillation counting procedures. These studies were followed up with the observation that during activation of the dormant embryos the rate of glucose oxidation increases and that this occurred whether the process was *in vivo* with estrogen or *in vitro* (Torbit and Weitlauf, 1974, 1975). Although these studies also suffered from potential questions about permeability and specific activity of endogenous pools, they raised the possibility that decreased energy production might lead to a reduction in synthesis of macromolecules and ultimately, the metabolic quiescence that seems to be responsible for the developmental arrest associated with delayed implantation. This scheme would implicate the regulation of energy production as a prime control in development of preimplantation embryos. Two other observations have tended to reinforce this idea: (i) Nilsson and his co-workers (Nilsson *et al.*, 1982) measured utilization of  $\text{O}_2$  by delayed implanting embryos and found it to be decreased. Although this might have been expected from the studies showing reduced oxidative utilization of glucose, it was significant because he measured absolute consumption of  $\text{O}_2$  and did not depend on the use of radioisotopes. This approach, then, is not open to questions about specific activity, permeability, etc. In the same report it was also noted that cytochrome oxidase activity was decreased in delayed implanting embryos and suggested that the lower oxidative utilization of glucose is due to a reduced capacity for oxidative phosphorylation and leads to decreased energy production. (ii) Embryos do not develop beyond the blastocyst stage if glucose is missing from the medium (Wordinger and Brinster, 1976; Naeslund, 1979; VanBlerkom *et al.*, 1979). It has been suggested that this may be equivalent to embryonic diapause *in vitro* and that restriction of glucose in the uterine lumen might actually lead to the arrested development in delayed implantation. Again the underlying idea here is that a lack of energy production is responsible, at least in part, for the quiescence associated with delayed implantation.

Although it seems clear from some of this work that utilization of glucose is reduced in delayed implanting embryos, two questions that need to be answered are: (i) what step in the metabolic scheme for glucose is regulated?, and (ii) whether the associated reduction in energy production is responsible for the decrease in macromolecular synthesis?

*Glycolysis and oxidative phosphorylation*

The observation by Menke and McLaren (1970a,b) that oxidative utilization of glucose is decreased in delayed implantation, along with the report by Nilsson and his co-workers (Nilsson *et al.*, 1982) that there is a reduction in cytochrome oxidase activity, suggests that oxidative phosphorylation is limited in delayed implantation. Further, it has been proposed that it is the resulting inability of the embryos to generate sufficient amounts of energy that leads to the reduction in macromolecular synthesis and the characteristic developmental arrest (Nilsson *et al.*, 1982). To test this hypothesis, experiments were undertaken in our laboratory to measure the capacity for citric acid cycle activity and oxidative phosphorylation in intact delayed implanting mouse embryos (Nieder and Weitlauf, 1983). (i) Dormant and reactivated embryos were recovered and incubated with 2-[ $^{14}\text{C}$ ]-pyruvate; evolved  $^{14}\text{CO}_2$  was trapped, and the amount of radioactivity was measured. After correcting for differences in embryonic mass, it was found that the ability to utilize pyruvate oxidatively was equal in the two types of embryos ( $74 \pm 11$  and  $72 \pm 9$  pmol  $\text{CO}_2/\text{h}/\mu\text{g}$  respectively). This result demonstrated that failure of delayed implanting embryos to oxidize glucose is not due to deficiencies in the citric acid cycle or oxidative phosphorylation and suggests either uptake of glucose or enzymatic regulation in the Embden-Meyerhoff scheme as possible control points. (ii) To avoid the questions raised by utilization of labelled substrates in testing the glycolytic pathway, embryos were recovered and placed *in vitro* in medium containing non-labelled glucose and the production of lactate (*i.e.*, in the presence of  $\text{O}_2$ ) over 2 h was measured. It was found that active embryos produced more lactate than delayed implanting embryos ( $92 \pm 5$ , and  $45 \pm 4$  pmol  $\text{CO}_2/\text{h}/\mu\text{g}$  respectively), thus confirming that uptake of glucose or the glycolytic pathway are restricted in the dormant embryos. (iii) The amounts of phosphofructokinase and hexokinase activity were measured in lysed embryos to determine if the levels of these primary regulatory enzymes could be responsible for reduced glycolytic activity. The levels of phosphofructokinase were not different in dormant and reactivated embryos ( $80.4 \pm 8.9$ ,  $72.5 \pm 6.3$  pmol/min/ $\mu\text{g}$  respectively). Interestingly, the level of hexokinase activity in the delayed implanting embryos was nearly 3 times that in the active embryos (*i.e.*,  $21.7 \pm 2.1$ , and  $6.5 \pm 1.2$  pmol/min/ $\mu\text{g}$  respectively), thus demonstrating that absolute amounts of these regulatory enzymes are not limiting in the dormant embryos. (iv) Transport of glucose into blastocysts was examined by measuring the initial rates of uptake and phosphorylation of [ $^3\text{H}$ ]-2-deoxyglucose at varying concentrations. The  $K_m$  for the substrate was not significantly different in the two types of embryos but surprisingly the  $V_{\text{max}}$  for dormant embryos was 2.2 times higher than that for the active embryos. This result demonstrates that the capacity for uptake and phosphorylation of glucose are not restricting in delayed implantation. (v) That glucose utilization by delayed implanting embryos is low in spite of the fact that the embryos have sufficient enzymes to maintain glucose uptake and glycolysis, as well as the capacity for normal citric acid cycle activity and oxidative phosphorylation of pyruvate, suggests that there is a negative regulatory control of glycolysis. To pursue this possibility lactate production was examined in anaerobic conditions designed to elicit, if possible; a Pasteur effect that would confirm the presence of negative allosteric feedback in the delayed implanting blastocysts. Embryos were incubated in medium with cold glucose

in the absence of  $O_2$  (*i.e.*, an atmosphere of  $N_2$ ); lactate production by dormant and active embryos over 2 h was increased several fold over that seen in the presence of  $O_2$  (*i.e.*, confirming a Pasteur effect) and was not different in the two types of embryos (*i.e.*,  $862 \pm 82$ , and  $906 \pm 75$  pmol/h/ $\mu$ g respectively). From this result it is clear that the two types of embryos have an equal capacity for glycolysis and that some form of negative allosteric feedback is responsible for regulating the Embden-Meyerhoff pathway. (vi) Several metabolites known to modulate the rate of glycolysis were then measured; ATP levels were found to be approximately 2-fold higher while ADP levels were 3-fold lower in delayed implanting (*i.e.*, compared to activated) embryos (*i.e.*,  $16.5 \pm 2.5$  and  $7.9 \pm 1.0$  pmol ATP/ $\mu$ g, and  $3.1 \pm 3.8$  and  $9.6 \pm 1.6$  pmol ADP/ $\mu$ g respectively). Hexosephosphates were also measured and found to be much higher in dormant than in activated embryos (*i.e.*, the level of combined glucose-6-phosphate and fructose-6-phosphate was  $5.25 \pm 0.56$  and  $1.14 \pm 0.22$  pmol/ $\mu$ g respectively). The buildup of these metabolites along with the high ATP:ADP suggest that phosphofructokinase is the regulated step in glycolysis and that the regulatory mechanism is the classic negative allosteric feedback described in numerous other systems. These results demonstrate that although the failure of delayed implanting embryos to utilize glucose oxidatively must result in a low rate of energy production, it is not responsible for the characteristic reduction in synthesis of macromolecules. On the contrary, it appears that reduced demand for energy is a result of decreased macromolecular synthesis and that the failure to utilize glucose oxidatively is a consequence of the resulting energy excess.

### Comment

Although it has been possible to elucidate some of the control mechanisms for glucose metabolism in 2-cell mouse embryos and delayed implanting blastocysts, their significance is not clear. Indeed, since the embryos at both of these stages have a high ATP:ADP and are probably bathed in pyruvate (Nieder and Corder, 1983) in the reproductive tract anyway, it is not likely that regulation of glycolysis, the availability of glucose, or changes in the capacity to utilize various metabolic intermediates would have important roles in controlling development of preimplantation embryos. If the great question in development is what regulates differentiation, the question in preimplantation biology must be how can the mother cause differentiation of the embryo to stop and restart at a later time as occurs with delayed implantation? The implication of the work reviewed above is that the mechanism probably involves control at the level of embryonic transcription or translation and not inhibition of oxidative phosphorylation or glycolysis, or restriction of energy substrates in the uterine lumen.

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## Blastocyst attachment and morphogenesis of ectoplacental cone in mouse

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**Abstract.** Mouse blastocyst attaches on the antimesometrial side of the uterus through mural trophoblasts. Later the polar trophoblasts begin proliferation, and rapid multiplication towards the mesometrial side of the uterus occurs resulting in the formation of an excrescence designated as ectoplacental cone. The morphogenesis of ectoplacental cone, viewed *in utero*, initiates on day 6 *post-coitum* when microvilli of the trophoblast and the uterine epithelial cells are lost and as a result of this opposing membranes appear interlocked with each other. Soon following the invasion by surrounding trophoblasts the necrosis of the epithelial cells starts. Mitochondriae of the epithelial cells, at this stage, are shrunken and lack well defined cristae. Several leucocytes are seen at the site and few electron dense structures appear wedged between the trophoblasts and epithelial cells. At places the cell membrane is studded with the basement membrane of the uterine epithelium giving an impression of a bristle coated membrane. By day 7 *post-coitum* the basement membrane has almost disappeared leaving trophoblast cells to develop close contact with stromal cells. Collagen fibres appeared between the trophoblasts and the stromal cells, many large inclusions of high electron density representing engulfed necrotic epithelial cells are discernible. On day 8 *post-coitum* the ectoplacental cone is fully developed. Four types of trophoblast cells can be identified in it: (i) basal cells lying on the base of the cone, are polyhedral and compactly arranged. They have a large nucleus and well developed nucleoli, (ii) central cells forming the middle area of the cone are of two types; one contained several osmiophilic granules enclosing translucent area (eccentric) and a well developed golgi complex around the nucleus, while the other has many heterophagosomes, vacuoles and residual bodies and (iii) peripheral cells contained several pleomorphic structures resembling secondary lysosomes. Minute dense granules and band of microfibrils on the apical region of these cells are seen. Dense granules probably release lytic proteins at the site and microfibrils help in forming cytoplasmic projections.

**Keywords.** Morphogenesis; ectoplacental cone; early mouse embryo.

### Introduction

Implantation in the mouse (on day 4-5 *post-coitum* (pc); Theiler, 1972) is initiated through abembryonic (or mural) trophoblasts on the antimesometrial side of the uterus (Snell, 1941). These cells later are converted into non-dividing primary giant cells (Snell and Stevens, 1966). Meanwhile embryonic (or polar) trophoblasts, lying over the inner cell mass (ICM) continue to multiply and proliferate on the mesometrial side of the uterus resulting in the formation of an excrescence (day 8 pc) over the ICM, designated as the ectoplacental cone (EPC) or *träger* (Snell, 1941; Amoroso, 1958; Barlow and Sherman, 1972; Gardner *et al.*, 1973). Cone is reported to be formed from multiplication of trophoblast cells and not as a result of proliferation of cells of the ICM, and

there is a strong evidence to suggest that the inside cells of blastocyst develop into inner cell mass and outside into EPC (Gardner *et al.*, 1973; Ansell, 1975). The suggested functions of the cone cells are: disintegration of the apposing uterine epithelium, nourishment of the embryo, secretion of hormones and providing immunological protection to the developing embryo (Kirby, 1971; Billington, 1975).

Earlier studies, based on light microscopy have indicated two types of trophoblast cells in mouse EPC; the large secondary giant cells lying on the periphery and the small tertiary or symplasma cells forming the base of the cone (Snell and Stevens, 1966). This study, being first of its kind, mainly focusses on the ultrastructural changes in polar trophoblasts and morphogenesis of ectoplacental cone, which begins on day 6 pc in mouse.

### Materials and Methods

Virgin female albino mice were caged overnight with males of proven fertility and the presence of vaginal plug on the following morning was considered as day 1 of pregnancy.

On days 6, 7, and 8 of pregnancy, respectively, the animals were perfused with 2.5% glutaraldehyde through dorsal aorta. Pontamine blue dye was injected to day 6 pregnant animals prior to perfusion to bring about the visibility of the implantation sites which are otherwise not very distinct on this day of pregnancy. The implantation sites were carefully segmented, post fixed in 1%  $\text{OsO}_4$  and after passing through routine dehydrating process embedded in agar resin.

Specimens were oriented on a flat lid of a beem capsule in a way to get vertical sections (to the axis of uterine horn) of the embryo. The lower end of the capsule with cut bottom was placed over the lid, filled with resin and kept for polymerization (48 h). Serial sections were obtained for light microscopy on a Pyramitome (L.K.B.; Bromma, Sweden) and after every 50  $\mu\text{m}$  a section was examined till the accessibility of the implantation chamber become evident. Mesometrial side of the chamber was marked for ultrathin sections.

### Results

On day 6 implantation chamber appeared like a biconvex cavity enclosing embryo, embryonic trophoblasts and few abembryonic giant cells. The chamber was surrounded by dark uterine epithelial cells. Stromal cells, particularly at mesometrial end, developed sinuses around the chamber. The uterine epithelial cells formed a dome like structure over the polar trophoblasts, which started insinuating between the adjacent epithelial cells. The luminal surface of the epithelial cells appeared ruffled at this stage and contact with trophoblasts became more extensive.

The microvilli on the epithelial cells and polar trophoblast were usually lost following adhesion, resulting in the interlocking of apposing membrane. Leukocytes were frequently noticed at the site. At places usually electron dense structures, wedged in between the trophoblast-epithelial complex could be seen. Glycogen granules and

lipid bodies, some being quite large, were abundant. The epithelial cells contained numerous pleomorphic vesicles.

On day 7 all the epithelial cells appeared to have vanished and the remaining ones were on the way of being phagocytosed by invading trophoblasts. The cytoplasmic extensions of the trophoblast penetrated through neighbouring cells until they reached the basement membrane, which appeared to be undergoing complete disintegration. As a consequence of this trophoblast formed close contact with stromal cells.

Several large inclusions of high electron density representing engulfed necrotic epithelial cells were observed inside the trophoblast. Often a large vacuole filled with digested material or cell debris was noticed adjacent to the nucleus of the trophoblast. The maternal cells contained large fat droplets. Beneath these cells, and between the basement membrane and the stromal cells thick bundles of fibres representing collagen, occasionally accompanied by flocculent material, were present (figure 1).

Phagocytosis of epithelial cells, erythrocytes and leukocytes, sometimes oftenly escaped from endometrial capillaries appeared to be the regular feature of the trophoblast cells. Lysosome like structures were frequently noticed in these cells.

On day 8 EPC was fully formed. The implantation chamber enclosed the embryo, abembryonic trophoblastic giant cells on antimesometrial and the cone cells on mesometrial end. The cone appeared porous as irregular strands of cells formed interstices between which maternal blood cells flowed (figure 2).

The entire cone region, being a preplacental component of a developing embryo could be divided into three areas (figure 3A, B): (i) the basal area containing compactly arranged proliferating cells (trophoblasts 1), (ii) the middle area having two types of cells, one (trophoblasts 2) with several osmiophilic granules and the others (tropho-

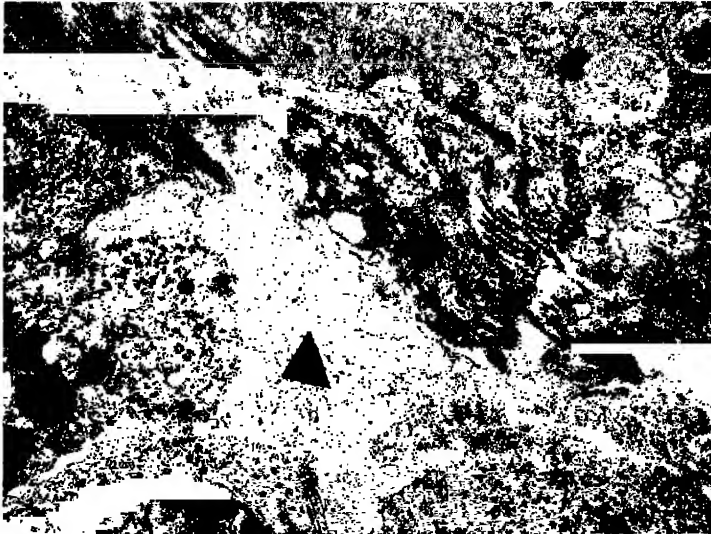


Figure 1. Micrograph showing bundles of collagen fibres (arrow) below the basement membrane of the uterine epithelium. Lower side is apical cytoplasm of trophoblast and upper side is maternal cell. Flocculent material may be seen at the site (arrow head).  $\times 2500$ .



**Figure 2.** Survey micrograph showing cells of inner cell mass (arrow) and of basal area (arrow head) of the cone. Cells of visceral endoderm (E) and uterine epithelium (U) are on extreme right and left respectively. Note lymphocyte (L) and a thrombocyte (T) near the uterine epithelium.  $\times 1600$ .

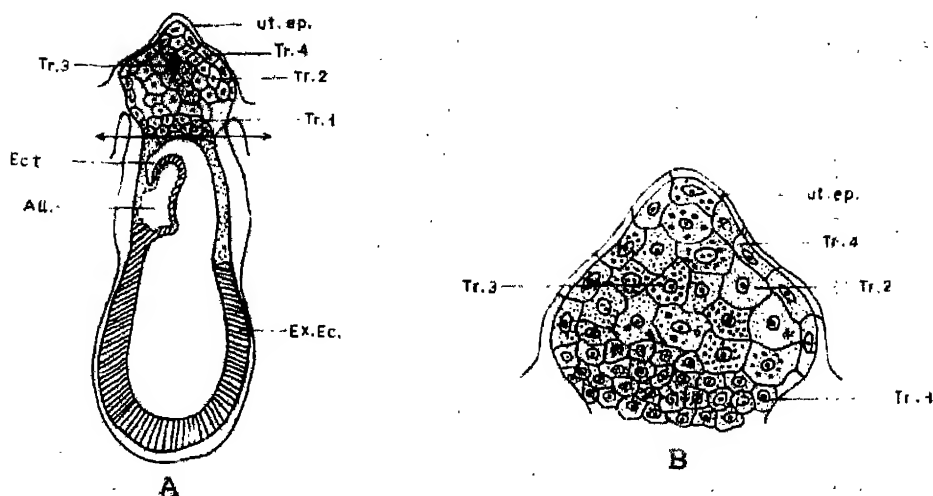


Figure 3. Schematic diagram showing relationship between ectoplacental cone and egg cylinder (A) magnified cone area (B). Sinuses have not been shown in it. (All—allantois; Ect—ectoderm; Ex.Ec—extra embryonic ectoderm; Tr. 1 to Tr. 4—trophoblast cells; ut. ep.—uterine epithelium).

blasts 3) with heterophagosomes, and (iii) the peripheral area containing relatively large and mostly elongated cells (trophoblasts 4) enclosing many lysosomes.

#### Basal area

Cells of this area being polyhedral in shape were joined with neighbouring cells through cytoplasmic extensions (figure 2). Nucleus, oval, spherical or sometimes kidney shaped, was very prominent having a large dense nucleolus, sometimes two or three nucleoli showing intranucleolar spaces (figure 4). Cytoplasm contained large number of ribosomes, generally in the form of rosettes or polysomes. Mitochondria were mostly discoidal containing many cristae and translucent matrix. Profiles of granular endoplasmic reticulum were sparsely scattered.  $\beta$ -Glycogen granules, at the supra nuclear position, were in abundance (figure 4). These features appear to be the characteristics of cytoplasmic growth in a proliferating cell (Batten and Haar, 1979). The appearance of intranucleolar spaces or vacuoles in nucleoli manifests the rapid turn over of the nucleoproteins (Van Blerkom *et al.*, 1973).

#### Central area

Cells of this area were of two types:

(1) *Trophoblast 2*: Nucleus of these cells were oval containing dark patches of heterochromatins. Nucleoli were relatively condensed. Large number of membrane bound osmiophilic granules ( $0.5 \mu\text{m}$ ) enclosing characteristic translucent area, mostly eccentrically situated were noticed in the cytoplasm of these cells (figure 5). Within

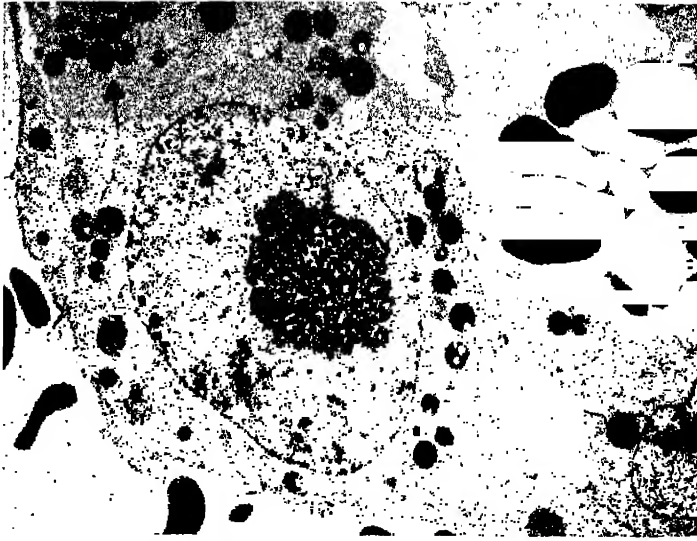


Figure 4. A cell of the basal area. Note  $\beta$ -glycogen granules (arrow) at supra nuclear position and intranucleolar spaces or vacuoles in nucleoli and a kidney shaped nucleolus.  $\times 29000$ .

these translucent areas several fine scrolls were noticed. In addition golgi complex was found to be well developed in these cells.

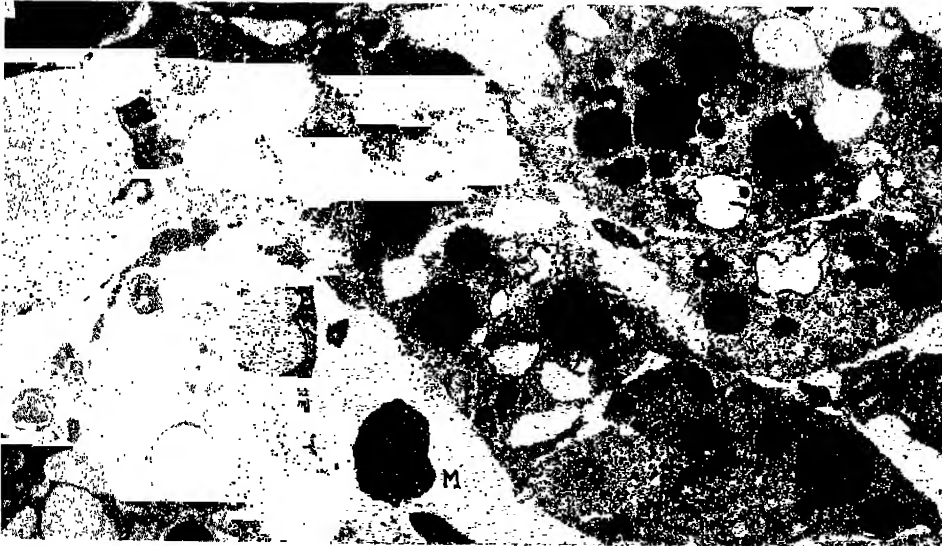
Such type of granules appear to be secretory in nature, since microstructures with similar morphology have been noticed in the theca lutein cells of the ovary and in the pituitary (Sandborn, 1976). Perhaps this type of trophoblasts are preferentially involved in secretion of hormones and steroids at preplacental stage.

(2) *Trophoblast 3*: These cells are of irregular size and dimensions. The nucleus is



**Figure 5.** Cell of the central area (Tr. 2). Note several osmiophilic granules (arrow) enclosing translucent area (eccentric). In sinuses maternal erythrocytes may be seen.  $\times 3200$ .

pleomorphic and covers major area of the cell. Many heterophagosomes having dense and granular substances, lipoid bodies and sometimes multilamellar structures inside were discerned (figure 6). Phagocytosis of erythrocytes, neutrophils and even lymphocytes were occasionally seen.



**Figure 6.** Cells of the central area (Tr. 3). Note several heterophagosomes (arrow) and vacuoles. Erythrocytes (arrow head) in different stages of disintegration are visible inside the cells. A wandering monocyte (M) may be seen.  $\times 1600$ .

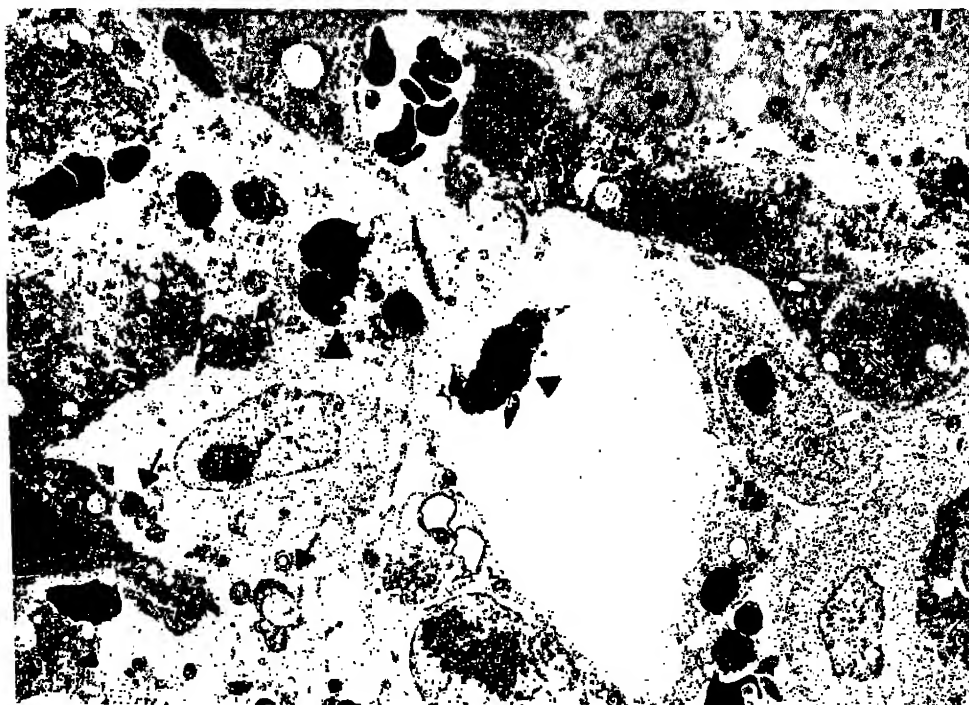


Structural entities of these cells reflect the pronounced phagocytotic activity which probably represents a second line of offence to peripheral trophoblasts. Mostly these cells phagocytose the necrotic epithelial cells.

#### *Peripheral area*

Cells (*trophoblast 4*) constituting this area formed a belt on the periphery of the cone. The geometry of these cells differed extensively, as they possessed large cytoplasmic extensions deeply penetrated into the maternal cells. The nuclei were mostly elongated. The most characteristic feature of these cells was the presence of several pleomorphic structures resembling secondary lysosomes (figure 7). Some of these contained an electron dense substance, while in others membranous structures were enclosed.

The apical region of the cytoplasm of these cells contained minute dense granules or structures having diffused outlines. Occasionally fine bundles of microfibrils organised in different manners were noticed in this area. Uterine remnants either projected into the cytoplasm of these cells (figure 8) releasing secretory material or the cytoplasmic extension of these cells invading into the maternal cells were often seen. Unusually large inclusions (0.5 to 1.0  $\mu\text{m}$ ) of irregular size and shape were seen at the site. Inside these a dense and amorphous substance was discerned (figure 8).



**Figure 7.** Peripheral cells (Tr. 4) facing maternal cells (upper and on left side; darker). Note several secondary lysosomes (arrow). Disintegrating neutrophils (arrow head) may also be seen, one lying freely in sinus.  $\times 1300$ .



**Figure 8.** Peripheral cell (lower) at the invading site. A microinclusion (arrow) of irregular shape having translucent area may be seen. Other microbodies enclosing ribosomes (arrow head) and RER are also visible. Maternal component (double arrow) is projected into trophoblastic cytoplasm. Note necrosis of the maternal cells. Upper left is part of a large lipid granules (L).  $\times 29000$ .

Well developed lysosomal system of these cells probably emerge as a causative factor for extensive phagocytotic activity (Potts 1968; Smith and Wilson, 1974; Billington, 1975). Engulfment of lymphocytes has been implicated in the prevention of immunological rejection of the embryo (Mulnard, 1970). The presence of minute dense granules in the apical area of these cells indicate that some secretory material, probably in the form of unspecified lytic proteins, is released at the site of invasion in order to cause degeneration of the maternal cells. Microfibrils form active component of the cytoplasmic extension and thus reflect on the migratory activity of a cell (Mehrotra, 1981; Enders *et al.*, 1981). Indeed, appearance of microfibrils in tumour cells has been implicated to be involved in invasive process (Tickley *et al.*, 1978).

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## Morphology of development in the primate: Blastocyst to villous placental stage

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**Abstract.** Examination of plastic-embedded rhesus monkey and baboon blastocysts through the implantation period has provided information on normal differentiation and development. The blastocysts show many features in common with non-primate laboratory animals, including differentiation of endoderm and its extension beyond the inner cell mass prior to implantation. However, there appears to be more cell death, and more aberrations in development. Implantation involves the adherence of trophoblast to healthy uterine luminal epithelial cells, and intrusion of syncytial trophoblast between these cells, followed by lateral expansion of the site of invasion prior to penetration of the uterine epithelial basal lamina. An amniotic cavity is formed within the inner cell mass, and is preceded by establishment of cell polarity. The definitive yolk sac is formed by an aggregation of endodermal cells adjacent to the inner cell mass. The trophoblast does not give rise to mesodermal cells, but some of these cells may be formed from endoderm prior to primitive streak formation. In both rhesus monkey and baboon, syncytial trophoblast taps the maternal vascular system relatively rapidly. In the baboon in particular large blood-filled spaces elevate the implantation site from the level of the endometrium at the stage of primary and secondary villus formation.

**Keywords.** Primate; blastocyst; implantation.

### Introduction

The cleavage and early implantation stages in mammals are extraordinary in that not only differentiation and delineation of cell lines are taking place, but at the same time the relationship to the maternal organism is undergoing rapid change. However, these fascinating stages are difficult to study in monovular species, such as most primates, with long gestation periods and relatively low fertility in the individual cycle (Short, 1979). Nevertheless considerable information is rapidly accumulating concerning the fine structure of these stages in a number of primate species, and the introduction of *in vitro* fertilization as a means of alleviation of certain types of infertility in the human is beginning to provide information from cleavage stages cultured *in vitro* (Edwards *et al.*, 1981). While both the number of species examined and the numbers of specimens from some of the stages remain small it is useful to summarize the features of differentiation of blastomeres of primates that have been studied to date, and the contribution of structural studies to our understanding of development of the principal fetal membranes.

## Materials and methods

The baboons and rhesus monkeys that were used in this study were housed at the California Primate Research Center, in accordance with standards of the Federal Animal Welfare Act and the Institute for Laboratory Animal Resources. Reproductive cycles were recorded, as were histories of previous pregnancies. Females were placed with males during the periovulatory period and evidence of breeding data recorded. In many cases blood samples were collected during the pre-ovulatory period, and serum estrogen (E) levels analysed to estimate the pre-ovulatory E peak. In the rhesus monkey, the day following the E peak was designated day 0 of pregnancy (Enders and Schlafke, 1981; Enders *et al.*, 1984). In baboons, the E peak occurs with greatest frequency approximately 3 days prior to the observed deturgescence of the sex skin (Shaikh *et al.*, 1982). Consequently the day of initial deturgescence was designated day 2 of pregnancy.

Preimplantation embryos from both rhesus monkeys and baboons were collected using surgical exposure of the uterus, followed by insertion of needles attached to introduction and collection cannula tubes (Hurst *et al.*, 1978; Enders and Schlafke, 1981). Intrauterine embryos from the baboon were collected using a nonsurgical transcervical approach (Pope *et al.*, 1982).

Early implantation stages from rhesus monkeys were collected following careful analysis of steroid levels during both the periovulatory period and the period of anticipated implantation (Enders *et al.*, 1984). If endocrine levels indicated a probable early implantation stage, the animals were anesthetized, and the abdominal aorta cannulated and infused with salt solution, then aldehyde fixative (Enders *et al.*, 1983). The reproductive tracts were then removed, and the animal was sacrificed by intravenous sodium pentothal.

Early postimplantation stages in the baboon were collected by Dr. Ross Tarara at the Institute of Primate Research, Nairobi, Kenya. In these animals the uterus was exposed at laparotomy, opened and the central portion of endometrium removed. This mass was separated to expose the luminal surface, which was examined for focal vascular regions. These implantation sites will be described in detail elsewhere.

Preimplantation embryos and implantation sites were retained in the aldehyde fixative (2% glutaraldehyde-2% formaldehyde in 0.1 M phosphate buffer, pH 7.3), rinsed in phosphate buffer, postfixed in 1% osmium in phosphate buffer, dehydrated and embedded in epoxy resin. Semithin sections (1  $\mu$ m) were stained with Azure B and observed with light microscopy. Thin sections were stained with uranyl acetate and lead citrate, and observed in Philips 400 or Zeiss 10 electron microscopes.

Early human implantation sites were described by Hertig *et al.* (1956). This material is in the Carnegie Collections, currently housed at Davis, California, USA. The earliest stages in this collection provided the reference material for this species during the course of these studies.

## Observations

The morula stage of primate development largely resembles that of other mammalian species, but with some modifications (Enders and Schlafke, 1981). The general shape of

the individual blastomeres does not change from the basic spherical shape until later than in the rodent (rat and mouse 8-cell stage: Schlafke and Enders, 1967; Ducibella and Anderson, 1975). When some of these cells begin to adhere closely to one another (compaction), not all cells form such associations at one time. As a consequence compaction is less abrupt and dramatic than in some other species. The morula stage of the rhesus monkey appears to be somewhat more light-lucent than that of the baboon and human (Panigel *et al.*, 1975; Lopatta *et al.*, 1982). With Nomarski optics, the nucleoli can be seen in nuclei of most of the blastomeres at this stage, and evidence of one or two fragmented blastomeres, a common features in primate morulas, is readily seen. When the first small spaces that are precursors of the blastocyst cavity begin to appear between blastomeres of the morula, fine structural examination generally reveals 'primitive' junctional areas (regions of close association of cell membranes with some increased density of the inner leaflet, but no other apparent aspects of junctional complexes).

The cytoplasm of healthy blastomeres has distributed throughout it clusters of mitochondria, many still with the more primitive type of cristae (Hurst *et al.*, 1978). At this stage the few strands of granular endoplasmic reticulum are often closely associated with the mitochondria (figure 1). Polyribosomes are still relatively sparse, and large areas of cytoplasm are consequently devoid of organelles. When the blastocyst cavity first becomes complete the inner cells constituting the inner cell mass (ICM) are confined to about a third of the sphere. By this stage the complete series of junctional types is present between trophoblast cells, including apical junctional complexes with tight junctions, desmosomes and small gap junctions. Intermediate filaments are associated with the desmosomes and bundles of such filaments are conspicuous features of the cytoplasm.

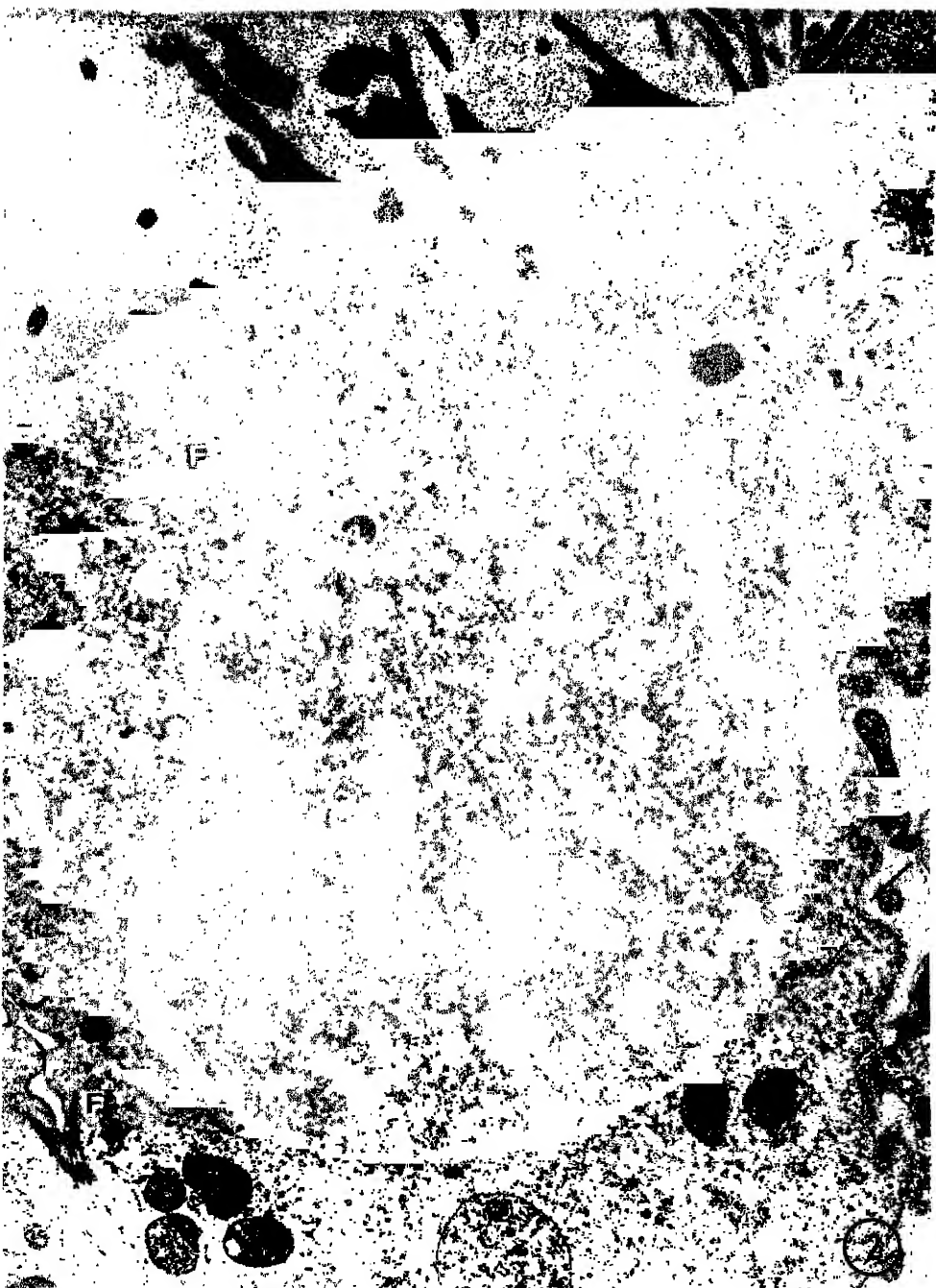
As the blastocyst increases in size a great deal of differentiation occurs (Enders and Schlafke, 1981; Mohr and Trounson, 1982). Although azonal blastocysts of the baboon have not been described, rhesus monkey and human blastocysts show trophoblast that has developed a series of apical coated vesicles and tubules, more numerous polyribosomes, some endoplasmic reticulum and the beginnings of a basal lamina (figure 2). This last structure, however, does not extend under the polar trophoblast but instead is continuous with the basal lamina of the epiblast. The junctional complexes are well developed at this stage; in particular gap junctions are numerous and extensive, and link all three cell types: trophoblast, epiblast and endoderm. The endoderm lacks a basal lamina, but forms a distinct layer beneath the ICM and extending beyond it prior to implantation. The cytology of the endodermal cells is similar to that of other species in having branched granular endoplasmic reticulum, with a distinct internal content.

All blastocysts examined have some evidence of autolysis, as indicated by the presence of vacuoles containing cell debris. Some also show individual isolated cells that lack junctional contacts and generally do not display the same degree of differentiation as other cells in the blastocyst.

Prior to implantation the trophoblast of some blastocysts develops long projections in addition to microvilli (figure 2). In the earliest implantation site of a rhesus monkey examined by electron microscopy, syncytial trophoblast has developed near the ICM, and processes from one mass of syncytium pass between epithelial cells to which the mass is adherent (Enders *et al.*, 1983). Initially no trophoblast penetrates the uterine



**Figure 1.** Normal (right) and abnormal (left) blastomeres in a morula from a baboon. Note the cluster of mitochondria towards the center of the abnormal blastomere. The mitochondria and endoplasmic reticulum (arrows) are distributed in the normal blastomere. The surface of the morula is in the upper left.  $\times 16,300$ .



**Figure 2.** Mural trophoblast from an azonal blastocyst of the rhesus monkey. Note the microvilli and irregular protrusion of the surface membrane, and the coated vesicles between and below the microvilli. Bundles of intermediate filaments (F) are present in the apical region of the cell, as well as near the desmosomes. A basal lamina (arrow) is barely visible underlying the trophoblast cells.  $\times 15,500$ .



epithelial basal lamina in the rhesus monkey; consequently the implant site expands in the plane of the uterine luminal epithelium.

Trophoblast in early implantation shows quite a bit of variation in structure. The abembryonic cytotrophoblast is initially unchanged. The cytotrophoblast adjacent to the implantation site proliferates, forming cells with multiple processes towards the syncytial trophoblast. The syncytium itself has regions where nuclei are aggregated, to the exclusion of most other organelles, and other regions rich in organelles.

Just prior to penetration into the stroma and subsequent to this, some of the syncytial trophoblast develops clefts and shows pronounced polarity (figure 3). The cleft areas constitute the microvillous apical surface of the syncytial trophoblast. Baboon implantation sites, from stages after epithelial penetration, also show similar clefts. When trophoblast encounters maternal vessels, in the rhesus and baboon, trophoblast seems to be able to penetrate between endothelial cells. This appears to be a later phenomenon in the human. The human in early stages, shows masses of syncytium with polyploid nuclei, a relatively rare occurrence in the other two species, and peripheral small nuclei of unknown origin. In both rhesus and baboon blood liberated from maternal vessels forms non-coagulating lakes within the syncytial trophoblast. In the baboon, in particular, the lakes rapidly enlarge, temporarily everting the implantation site so that it mushrooms from the surface of the endometrium. The evaluation of implantation sites in the rhesus is less extensive, and by this time a secondary implantation site has formed on the abembryonic trophoblast surface. Although the human is estimated to implant three days earlier, the formation of large blood-filled lacunae is slower so that all the three species achieve such structures at approximately day 11.

At the beginning of implantation, the ICM of the rhesus is a slightly flattened disc of epiblast covered by endoderm, which extends at least a third around the sphere of the blastocyst. The epiblast cells adjacent to endoderm are slightly larger than those contiguous with the cytotrophoblast. The first evidence of differentiation within the epiblast is the formation of 'apical' junctions in the center of the disc of epiblast cells. By day 10.5, fluid accumulates adjacent to the apical ends of these cells, giving the first evidence of an amnionic cavity. The cells that will form the amnion are largely separated from the cytotrophoblast except at the very center, where the basal lamina of the amnion cells loops back over the cytotrophoblast, thus leaving some cells contiguous. The cells forming the amnion are smaller than those forming the embryonic plate. With continued development, in all the three species, the amnion becomes separated from the cytotrophoblast and the embryonic shield extends in diameter as a tall columnar epithelium with occasional evidence of pseudostratification.

Before there is any thickening of the future posterior end of the embryonic shield, a few cells separated from the endoderm are present between it and the basal lamina of the trophoblast. The endodermal cells underlying the embryonic shield form a poorly defined cluster, which becomes separated from the primary yolk sac. The cluster of cells cavitates forming a secondary yolk sac. In one instance, in the baboon, the embryo developed upside down and the secondary yolk sac appeared as a completely separate cluster of cells between the embryonic plate and trophoblast.



**Figure 3.** In the centre of the micrograph is a cleft in the syncytial trophoblast. The formation of these microvillus-lined spaces demonstrates a change in the syncytial trophoblast to a thin polarized layer lined by the type of syncytium that will enclose the maternal blood spaces. Implantation site on day 10.5 of gestation, rhesus monkey.  $\times 9,200$ .

## Discussion

It is apparent from examination of preimplantation embryos of the human, baboon and rhesus monkey, that considerable disintegration of blastomeres occurs during development. From the observations of vacuoles containing cell debris in normal blastocysts, it seems likely that some autophagy can be tolerated. However, many cleavage stages seem to become progressively abnormal. This problem is countered in human *in vitro* fertilization systems by introducing as many as three cleavage stages into each uterus. However it would be better to be able to judge the potential success of the *in vitro* fertilized ova prior to reintroduction.

One of the more common features of blastomeres in cleavage stages with numerous pycnotic cells, in unfertilized ova and in isolated cells is the aggregation of mitochondria around the nuclei rather than being in dispersed groups. It is not yet known whether such blastomeres can recover from this abnormal configuration, or what percentage of cells in the morula can fail without compromising the conceptus. There does not appear to be any pattern as to where cell death occurs in the blastocyst. Consequently it appears that the blastocyst must have a mechanism for compensating for cell death in the trophoblast and for initially variable numbers of cells in the ICM. This would suggest that the type of compensation for reduced numbers shown experimentally in laboratory (Snow and Tam, 1979), and domestic animals is probably a common feature of primate development.

The regional variability as well as variation in time of development of syncytial trophoblast is striking. One would like to know what causes initial formation of syncytium. In the absence of cell boundaries, how can one region of syncytium be different from another? Clearly the syncytium that comes to form the lining of blood spaces is very different from that involved in the initial penetration of epithelium in the rhesus monkey, or from that involved in stromal penetration in the human. There is also variation in the nature of trophoblast invasion in the different species. The trophoblast of rhesus and baboon appears to tap maternal vessels more readily. In the human, while trophoblast initially penetrates further into the stroma, it is seemingly slow in tapping maternal vessels, and the earliest lacunae formed are more labyrinthine.

The formation of amnion in the rhesus is by cavitation. At the time that this is initially taking place endodermal cells are cuboidal in the region of the ICM. They become less closely associated with the embryonic plate prior to the formation of the secondary yolk sac. The cells that become separated from the endoderm and are situated between it and the trophoblast have been called endodermal reticulum (Luckett, 1974). The eventual fate of these cells is not known but they are in the position of forming mesodermal constituents, which suggests that some of the extraembryonic mesoderm may come from this source rather than primitive streak.

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## Biochemical interactions between blastocyst and endometrium in the large domestic animals

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**Abstract.** In pigs, the blastocyst begins to elongate from a sphere to a long filamentous thread around day 10.5 of pregnancy. At about this time the endometrium secretes large quantities of protein into the uterine lumen. The synthesis of this material which is believed to be required for nutritional support of the conceptus is under the control of progesterone. The release of secretory protein appears to be triggered by the production of estrogens by the elongating blastocyst. Blastocyst estrogens are also involved in the phenomenon of maternal recognition of pregnancy in swine, and their interaction with the maternal system, by a mechanism as yet unknown, prevents a return to reproductive cyclicity. Maternal recognition of pregnancy in the sheep and cow occurs at around the time of blastocyst elongation. Here estrogens do not appear to be involved, and protein products secreted by the conceptus have been implicated. One product of the sheep, ovine trophoblast protein-1, which is produced only during a brief period (days 13-21) of pregnancy, has been purified. It appears to be a hormone whose target tissue is the uterine endometrium.

**Keywords.** Uterus; endometrium; blastocyst; secretions; proteins; estrogen; progesterone; pregnancy; corpus luteum.

### Introduction

Pregnancy depends upon a series of appropriate coordinated interactions between the developing conceptus and the mother (Bazer and First, 1983). The maternal system, for example, must afford an appropriate fluid medium for fertilization within the oviduct. In the case of large domestic animals, where ectopic pregnancies do not occur, only the uterus appears capable of providing the micro- and macromolecular environment and possibly other factors, such as hormones, that are required for continued conceptus development beyond the blastocyst stage. The uterus must also limit the degree of blastocyst invasiveness and the extent of placentation. In turn, signals must originate from the conceptus to protect the corpora lutea (CL) from regression and to prevent the return to reproductive cyclicity. In addition, uterine secretory activity must be maintained, blood flow to the endometrium regulated, immunological privilege of the fetal allograft achieved and mammary gland development promoted. Chemical signals between the conceptus and mother are also responsible for initiating parturition.

In this paper we shall review some of the interactions that occur between the blastocyst and uterine endometrium in early pregnancy of pigs, sheep and cattle. We shall concentrate on those events that occur around the time the blastocyst starts to

Abbreviations used: CL, Corpora lutea;  $E_1SO_4$ , estrone sulphate;  $E_1$ , estrone,  $E_2$ , estradiol;  $E_3$ , estriol;  $E_2V$ , estradiol valerate;  $PGF_{2\alpha}$ , prostaglandin  $F_{2\alpha}$ .

elongate, because this period coincides with a series of additional events which appear to be crucial for maintenance of pregnancy. In the pig, for example, it is the time when the maternal endometrium begins to release large quantities of progesterone-induced secretory material into the uterine lumen (Geisert *et al.*, 1982a). In all the three species, it coincides with the period during which the phenomenon of maternal recognition of pregnancy occurs (see below). Finally, as the blastocyst begins to expand, it also begins to become active in the production of its own secretory protein products and in steroid metabolism (Flint *et al.*, 1979).

### Conceptus elongation

In swine, sheep and cattle, blastocysts undergo a dramatic morphological sphere-to-filamentous transition some days prior to their firm attachment to the uterine wall and to the expansion of the allantois (Bazer and First, 1983). Elongation results in a major increase in the surface area to volume ratio of the conceptus and a close association of the trophoctoderm (outer epithelial surface) with the maternal uterine endometrium. It is also around this stage of development that the process of maternal recognition of pregnancy occurs, a phenomenon that must result from a chemical signal passing from the conceptus to the maternal system to insure maintenance of CL function and continued progesterone production. In turn, progesterone is responsible for ensuring induction and synthesis of the endometrial secretions upon which the noninvasive conceptuses must rely for their growth and development. Indeed we have compared the secretions produced by the maternal uterus during its formation to a complex embryo culture medium (Bazer *et al.*, 1978). Elongation may be necessary in order to provide a sufficient area of trophoctoderm surface to allow uptake of nutrients and to facilitate the biochemical interactions between the conceptus and maternal uterus upon which pregnancy depends.

In swine, embryos enter the uterus at the 4-cell stage, *i.e.* 60 to 72 h after onset of estrus, reach the blastocyst stage by day 5, shed the zona pellucida between days 6 and 7 and then slowly expand to a 2 to 6 mm diameter sphere by day 10 (Perry and Rowlands, 1962). Between days 10 and 12 the conceptus changes from a sphere of up to 10–15 mm diameter to a tubular form (15 to 50 mm long by 1 to 2 mm wide) and finally achieves a long, thread-like filamentous form about 200 mm long and 0.5 to 1 mm wide (Geisert *et al.*, 1982b). These initial stages of blastocyst elongation occur very rapidly. Rates of 30 to 45 mm/h have been estimated, and the morphological transition apparently results from remodelling and not cellular hyperplasia. Indeed the amount of DNA scarcely changes during sphere to thread transition and the volume of the conceptus actually decreases. However, the surface area during the initial transition from a 10 mm sphere to a 200 mm  $\times$  1 mm thread, must increase several fold.

Pig conceptuses continue to elongate between days 12 and 16 to reach a final average length of about 800 mm each (Anderson, 1978). During these final stages of elongation mitotic activity is high as reflected by substantial increases in total RNA and DNA per conceptus (Geisert *et al.*, 1982b).

Ovine and bovine blastocysts also undergo elongation, but in a less dramatic fashion than in pigs, and they do not achieve as long a final length. In the ewe elongation begins

around days 12–13. They reach a length of ~ 70 mm by day 14 (Bindon, 1971) and 150 to 190 mm by day 15 (Chang and Rowson, 1965). In the cow, rapid blastocyst elongation is initiated around day 15, and they are > 50 mm in length by day 18 (Chang, 1952). In neither of these animals has the mechanism of the process received detailed attention, but we assume that the events occur in the progression similar to that described for pigs by Geisert *et al.* (1982b).

Data are not presently available to elucidate the underlying mechanisms responsible for the cellular remodelling that occurs during blastocyst elongation, although some of the ultrastructural changes have been described for the pig, and a model accounting for the patterns of cellular migration has been presented (Geisert *et al.*, 1982b), but will not be discussed further here. Significantly, the outer surface of trophoblast cells are densely covered with coated pits (Geisert *et al.*, 1982b), indicative of endocytosis, and the uptake of components of maternal uterine secretions has been demonstrated in this species (Fazleabas *et al.*, 1982; Chen *et al.*, 1975). It is also known that the elongating blastocysts are active not only in uptake of protein, but also in secretion of proteins (Godkin *et al.*, 1982a).

#### *Relationship between uterine secretory activity and the development of the uterine endometrium*

In animals such as the pig which possesses a diffuse, central-type, epitheliochorial placentation (Schlafke and Enders, 1975), there is no erosion of the uterine epithelium throughout pregnancy. Several cell layers, therefore, separate the blood supply of the mother from that of the fetal placenta. It is probably for this reason, therefore, that the pig conceptus receives any macromolecular constituents required for its survival in the form of secretions provided by the glandular epithelium of the maternal endometrium over which it lies (Amoroso, 1952). As anticipated, the pig uterus continues to secrete large quantities of macromolecular secretory components, often known as, histotroph, throughout pregnancy although the rates of secretion change in relation to the endocrine status of the mother (Basha *et al.*, 1979). These materials have been investigated in detail over the last decade, and several proteins have been characterized (Bazer *et al.*, 1981; Roberts and Bazer, 1980). Among these are uteroferrin, a purple coloured glycoprotein, which carries iron to the conceptus (Buhi *et al.*, 1982; Ducsay *et al.*, 1982; Renegar *et al.*, 1982), a retinol binding protein, presumably responsible for the transport of that fat soluble vitamin (Adams *et al.*, 1981), and a series of protease inhibitors (Fazleabas *et al.*, 1982). The functions of the latter are unclear but they may help control the activity of conceptus proteases and even limit the potential invasiveness of the pig conceptus. Alternatively, they may protect the histotroph itself from proteolytic destruction. All of the proteins described above are induced by the hormone of pregnancy, progesterone, although their release from the secretory epithelium may be partially controlled by estrogens produced by the conceptus (see below). Moreover, although estrogens alone do not induce specific uterine proteins, at low concentrations they act synergistically with progesterone to promote increased amounts of secretory protein production while in high amounts they inhibit the production of histotroph (Knight *et al.*, 1974; Roberts and Bazer, 1980). Finally, an



estrogen "primed" uterus is probably necessary to provide a necessary number of cells for a subsequent secretory response to progesterone.

The most complete description of the changes that occur in the endometrium of pigs during the estrous cycle and early pregnancy are those of Corner (1921). Detailed ultrastructural observations have also been made throughout pregnancy from day 30 onwards by Sinowatz and Friess (1983) and in early pregnancy by Geisert *et al.* (1982a). In general, these observations are consistent with the concept that the progestational uterus of the pig is highly secretory. Essentially the first 15 days of the estrous cycle and pregnancy have been observed to be quite similar (Corner, 1921). Mitotic activity in the surface epithelium was high around estrus, and the cells become more columnar as the cycle progresses. Mitotic activity in the glands was observed to be highest around days 5 to 6, but mitoses in the deeper glands were evident until about day 11. During the luteal phase the glandular cells gained the appearance of being active in secretion. Data from our laboratory have indicated that a synchronized release of secretions begins around day 11 of pregnancy, while in the nonpregnant conditions this release is somewhat delayed and more gradual (Geisert *et al.*, 1982a).

Between day 17 and next estrus (day 21) the surface and glandular epithelial cells gradually revert to an appearance characteristic of estrus. Presumably there is also a loss of cells from the epithelium and glands, but the patterns of cellular and glandular regression have not been well studied. By contrast, in the pregnant condition, the surface and glandular epithelium remains columnar in appearance, and the glands becomes increasingly branched and complex. Between days 30 and 60 of pregnancy the cells of the glands in particular develop massive whorls of rough endoplasmic reticulum (Sinowatz and Friess, 1983). Large secretory vacuoles containing densely staining material are present. These structures can be stained immunocytochemically for uteroferrin (Renegar *et al.*, 1982; T. W. Raub, R. M. Roberts and F. W. Bazer, unpublished results) and appear to empty their contents into the lumen of the glands where uteroferrin-positive material can also be detected. The glandular cells maintain this striking appearance until term (day 115).

In the sheep and the cow such a reliance of the conceptus on uterine secretory activity is believed to be much less than in the pig due to the development of placentomes (fused fetal cotyledon and maternal caruncle) which become evident soon after the chorioallantois (placenta) has spread over the available surface of the placenta (Hafez and Jainudeen, 1974). Development of the cotyledons on the placental side is presumably initiated by the presence of opposing maternal caruncles (King *et al.*, 1980). In these regions the uterine epithelium is eroded (Boshier, 1969). The placentomes are believed to be the major sites of gas and nutrient exchange after about day 30 of pregnancy. The caruncles, for example, receive over 80% of the maternal uterine blood supply in the sheep and the cotyledons over 90% of the placental circulation (Hafez and Jainudeen, 1974). In addition, when the placental surface area is decreased either by twinning or by surgical restriction the size of the placentomes increases to accommodate the growth needs of the fetus (Caton *et al.*, 1983). It is assumed that these structures are also major sites of exchange of endocrine information between the mother and conceptus. It is within the fetal cotyledons, for example, that placental lactogens are believed to be formed (Chen *et al.*, 1975; Martal and Djiane, 1977; Kensinger *et al.*, 1982) and where estrogen production takes place.

### *Initiation of blastocyst estrogen secretion by conceptuses*

Pig blastocysts begin to produce estrogens soon after day 10 of pregnancy (Flint *et al.*, 1979; Fischer, 1981; Geisert *et al.*, 1982a). The two reports from our laboratory (Fischer, 1981; Geisert *et al.*, 1982a) have shown that production begins at around the 10 mm spherical stage (days 10.5 to 11) just preceding the rapid transition from sphere to thread. Based on concentrations of estrone sulphate ( $E_1SO_4$ ) in maternal plasma, estrogen secretion by pig conceptuses is triphasic with major increases between days 10 and 12 (Stoner *et al.*, 1981), days 16 and 30 (Stoner *et al.*, 1981) and day 60 and term (Knight *et al.*, 1977). The mechanisms whereby conceptus estrogen production is regulated is not known, nor is much evidence available to indicate what functions these estrogens, particularly those formed during the later two phases, play during pregnancy, although they may regulate uterine secretory activity. In this review we shall be concerned only with estrogen production by early expanding blastocysts where some functional relationships between the conceptus and mother have been established.

Total recoverable estradiol ( $E_2$ ), estrone ( $E_1$ ) and estriol ( $E_3$ ),  $E_1SO_4$  and  $E_2SO_4$  in uterine flushings of gilts increase markedly as blastocysts begin to increase in size from ~ 5 mm spheres (at day 10) elongated filaments at day 12. These estrogens then decrease by day 14 (Geisert *et al.*, 1982a). A corresponding increase in utero-ovarian vein plasma  $E_1SO_4$  is observed during the day 10 to 12 period and is assumed to have originated from conceptus estrogen production (Stoner *et al.*, 1981).

Data concerning estrogen production by bovine or ovine conceptuses are very limited. Shemesh *et al.* (1979) reported that bovine blastocysts between days 13 and 16 produced small quantities of  $E_2$ , and Eley *et al.* (1979), have demonstrated metabolism of progesterone and androstenedione to estrogens. However, there is no evidence for a corresponding increase in utero-ovarian vein estrogens during this period. Steroid production by ovine blastocysts has not been established. Ellinwood *et al.* (1978) reported that  $E_1$  concentrations in maternal utero-ovarian vein were higher than in nonpregnant controls in the day 13 to 17 period, but Reynolds *et al.* (1982) were unable to show such differences.

Clearly, systematic studies for steroid production by ovine and bovine blastocysts have not been carried out. Such studies will be necessary if transient periods of estrogen production, as occur in pigs, are to be detected.

### *Estrogen-induced secretion by endometrial epithelium*

Geisert *et al.* (1982a) reported the accumulation of secretory vesicles in endometrial gland epithelium of the pig between days 10.5 and 12 of gestation. With onset of estrogen production by tubular and filamentous blastocysts between days 11 and 12 of gestation, there was a marked increase in total recoverable calcium, prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ),  $PGE_2$ , uteroferrin and protease inhibitor levels in uterine flushings. There were no significant changes in these same components in uterine flushings, collected from nonpregnant gilts during a corresponding time period. In these animals the release of secretory materials was delayed and occurred more gradually. However, administration of 5 mg estradiol valerate ( $E_2V$ ) to nonpregnant gilts on day 11 resulted in increases in total recoverable calcium, prostaglandin and total protein, uteroferrin and protease inhibitor activity by 24 h post-injection which were similar to those for

pregnant gilts with respect to quantitative values and temporal patterns of change of each component (Geisert *et al.*, 1982c). It has been proposed that blastocyst estrogens may be responsible for this synchronous release of secretory material. Rubin and Laychock (1978) have suggested that increased calcium activates phospholipase  $A_2$  which releases arachadonic acid from membrane phospholipids. This event, in turn, triggers exocytosis of secretory vesicles and formation of prostaglandins *via* the arachidonate pathway. Conceivably blastocyst estrogen causes the initial release of calcium from the plasma membrane leading to the above cascade of events. Pietras and Szego (1979a, b) have reported that a site of estrogen action in the uterus of the rat is the plasma membrane and that there is a very rapid cytological response to the steroid (Rambo and Szego, 1983). Alternatively, the secretory mechanism may involve intracellular estrogen receptors.

A very similar series of events has been reported in the cat by Bareither and Verhage (1980). There, however, it is estrogen which induces the accumulation of intracellular secretory product, and progesterone which triggers rapid exocytosis of this material from secretory vesicles. In the roe deer there is also a marked accumulation of secretory vesicles in uterine epithelium during the period of embryonic diapause (July to December) (Aitken, 1979). These vesicles undergo exocytosis in association with with the termination of delayed implantation which is accompanied by an increase in calcium and  $\alpha$ -amino nitrogen in the uterine lumen. There is also evidence that the blastocyst of the roe deer begins to produce estrogen prior to the termination of the delay period. In the preceding examples, therefore, steroid hormones synthesized by the blastocyst cause the release of secretory products from the maternal endometrium which are presumably required for continued growth and development of the conceptus.

#### *Blastocyst estrogens and maternal recognition of pregnancy in pigs*

The life span of the CL determines the duration of progesterone production and therefore the period of histotroph production by the uterine endometrium. It is therefore essential for continuation of pregnancy that the conceptus should provide a "signal" which will result in the maintenance of the CL and thus allow for the establishment of pregnancy. This period of early pregnancy (days 11–14 in swine) when the conceptus acts to prevent CL regression is recognized as the period in which "maternal recognition of pregnancy" occurs. In the domestic farm animals it is now generally agreed that PGF<sub>2 $\alpha$</sub>  produced by the uterine endometrium is the luteolytic agent which will cause CL regression and the cessation of progesterone production (Bazer *et al.*, 1981a). In the pig, therefore, the conceptus must produce some substance or substances that directly or indirectly prevent the luteolytic effects of uterine PGF<sub>2 $\alpha$</sub> . The theory has been developed that estrogens produced by the embryo prevents PGF<sub>2 $\alpha$</sub>  release from the uterine endometrium into the uterine venous drainage where it would gain access to the CL and cause luteolysis (Bazer and Thatcher, 1977). This theory has been discussed in detail elsewhere (Bazer *et al.*, 1981). In support of this concept it has been noted that whereas utero-ovarian vein plasma concentrations of PGF<sub>2 $\alpha$</sub>  and metabolites are elevated during the period of luteolysis in non-pregnant gilts, no significant changes were noted at equivalent time periods in pregnant gilts. Similarly if

nonpregnant gilts were treated with estradiol valerate (5 mg/day) on days 11 through 15 after onset of estrus, the increase in PGF levels did not occur (Frank *et al.*, 1977). Moreover these animals became pseudopregnant in the sense that luteal function along with continued endometrial secretory activity was prolonged on average for more than 100 days.

The precise mechanism whereby blastocyst estrogen prevents the secretion of PGF<sub>2 $\alpha$</sub>  in an endocrine direction (*i.e.* towards the uterine vasculature) remains unknown. One possibility is that PGF<sub>2 $\alpha$</sub>  is diverted in an exocrine direction (*i.e.* towards the uterine lumen), since PGF<sub>2 $\alpha$</sub>  concentrations are high in uterine flushings of pseudopregnant and pregnant gilts in the day 11 to 14 period (Geisert *et al.*, 1982a). The concept that PGF<sub>2 $\alpha$</sub>  is the luteolytic agent in swine and that estrogens are luteostatic will not be reviewed in further detail here since the concepts have been discussed thoroughly elsewhere (Bazer *et al.*, 1981). Nevertheless, it should be emphasized that other interpretations of the results are possible. One is that the blastocyst produces some other substance, such as a protein, which acts locally on the endometrium, which in turn responds by producing estrogens or other substances to reduce output of PGF<sub>2 $\alpha$</sub>  into the utero-ovarian vein.

#### *Maternal recognition of pregnancy in the sheep and cow*

In the sheep, the CL regresses at the end of a 16–17 day estrous cycle as the result of the action of a uterine luteolysin which, as in the pig, is probably PGF<sub>2 $\alpha$</sub>  (McCracken *et al.*, 1972). The PGF<sub>2 $\alpha$</sub>  is again believed to be produced by the uterine endometrium and transported to the ovary in a local fashion. The presence of viable conceptuses within the uterus prevents CL regression, but the nature of the blastocyst product which prevents CL regression is unknown, but is thought not to be estrogen. However, a transient period of estrogen production has not yet been definitively ruled out.

Embryo transfer experiments with sheep have shown that a conceptus must be present in the uterus by days 12–13 of pregnancy for CL to remain functional and for pregnancy to be maintained (Moor, 1968). It was also shown that conceptus homogenates from days 14–15 of pregnancy maintained luteal function when infused into the uterus of nonpregnant, day 12, recipient ewes (Rowson and Moor, 1967). Extracts derived from day 25 sheep conceptuses or homogenates from day 14 pig conceptuses were ineffectual as was the transfer of heat-treated days 12–14 conceptuses. Repeated daily infusions of days 14–15 sheep homogenates were more effective than a single infusion at day 12. Similar results were obtained by Martal *et al.* (1979) who reported that daily intrauterine injections of extracts of days 14–16, but not days 21–23 conceptuses into day 12 recipients were sufficient to extend luteal function for up to several months in over one-half of recipient ewes tested. The active component was proteinaceous and was called trophoblastin. Although it is not completely resolved whether factors from the conceptus can affect the CL directly (Godkin *et al.*, 1978), most available evidence is consistent with the view that the conceptus proteins responsible for maternal recognition of pregnancy must be introduced into the uterine lumen, where they appear to exert their effect (Moor, 1968; Ellinwood *et al.*, 1979). Presumably, their main site of action is on the endometrium which, in turn, may

respond by altering the production or release of  $\text{PGF}_{2\alpha}$  or alter the ability of  $\text{PGF}_{2\alpha}$  to cause luteolysis.

Considerably less is known about maternal recognition of pregnancy in the cow. Northey and French (1980) reported that pregnant cows from which embryos were removed on day 17 and 19 had interestrus intervals of  $25 \pm 1.2$  and  $26.2 \pm 0.6$  days compared with those having embryos removed on day 13 ( $20.2 \pm 0.8$  days) or not mated 20 to 21 days. The nature of the active substances remains unknown, but by analogy with the sheep it may be a blastocyst protein.

### *Polypeptides released by incubated blastocysts*

**Pigs:** Pig conceptuses secrete a number of proteins, beginning as early as day 10.5 of pregnancy (Godkin *et al.*, 1982a). These results were obtained by culturing conceptuses of increasing age and development in a culture medium containing radioactive amino acids. Protein synthesis continued for at least 24 h when the experiments were terminated. Tissue proteins, as well as secreted products, were analyzed by two-dimensional polyacrylamide gel electrophoresis and detected by fluorography. Proteins in the medium were also analyzed by gel permeation and ion-exchange chromatographic methods.

The proteins released into the medium were relatively few and were clearly distinguishable from the major tissue proteins. We believe that they represent secretory products (Godkin *et al.*, 1982a). The pattern of these secreted proteins changed markedly in both a qualitative and quantitative manner with increasing age of the conceptuses. Between days 10.5 and 12 a group of acidic low molecular weight proteins ( $\text{pI}$  5.6 to 6.2;  $M_r$  20 to 25 K) predominated. At days 13 and 16 the small acidic proteins were still present but the major product was a basic protein ( $M_r$  35 to 50 K;  $\text{pI} \sim 8.0$ ). With time this protein also disappeared from the gels and was not a detectable product of day 20 to 35 chorionic tissue.

In addition to the above, the cultured pig conceptuses throughout the elongation stage produce a glycoprotein which does not enter polyacrylamide gels. This molecule is 50% by weight carbohydrate and of very high molecular weight ( $\sim 700,000$ ) (Masters *et al.*, 1982). Preliminary experiments have shown that this glycoprotein was not produced by day 10.5 spherical blastocysts and that it was released only during the elongation phase (W. Grey, F. W. Bazer and R. M. Roberts, unpublished results).

The function of the above proteins produced by the pig blastocysts remain unknown although it is tempting to speculate that their appearance reflects some specific but as yet mysterious role in the maintenance of pregnancy.

**Sheep:** Sheep conceptuses between days 13 and 23 also secrete a variety of proteins into the medium when they are cultured *in vitro* (Godkin *et al.*, 1982b). Using a modified Eagle's Minimal Essential medium supplemented with L-[ $^3\text{H}$ ]-leucine as a polypeptide precursor it was found that the release of proteins was linear for at least 24 h and that day 16 blastocysts could convert up to 8% of the radioactivity supplied into non-dialysable macromolecules in the medium. Two-dimensional polyacrylamide gel electrophoresis revealed that at day 13 there was only one major product produced.

Initially this protein was called Protein X (Godkin *et al.*, 1982b), but we have since named it ovine trophoblast protein-1 (or oTP-1) (Godkin *et al.*, 1981a and 1984). Ovine oTP-1 consists of three closely similar isoelectric species (pI's around 5.3 to 5.7) each with molecular weights of 17,000 (Godkin *et al.*, 1982b). Between days 14 and 21 additional proteins were detected. One of these was of high molecular weight ( $> 660,000$ ) and did not appear on the two-dimensional gels. Its properties have been discussed in detail elsewhere (Masters *et al.*, 1982). Ovine oTP-1, however, continued to predominate as a major secreted product until day 23 when it could no longer be detected (Godkin *et al.*, 1982b). Explants of chorion from day 30 of pregnancy failed to secrete oTP-1. It was shown that oTP-1 was a product of the trophoblast and not of the yolk-sac by dissecting out the two tissues from day 16 conceptuses and culturing them separately.

The ovine oTP-1 is produced in significant amounts by conceptuses in the middle of the day 13–21 period. For example, day 14 to 16 conceptuses release 50 to 100  $\mu\text{g}$  of oTP-1 in 24 h, although very little of the protein is detectable in the tissues themselves. Because it was a major protein product of the conceptus it was possible to purify oTP-1 from culture medium by a two step procedure involving successive DEAE-ion exchange chromatography and gel filtration on a column of Sephacryl S-200 (Godkin *et al.*, 1982b). Because oTP-1 and some of the other proteins appear to be produced transiently during the critical day 13 to 21 period, it has been suggested that they may play a role in maternal recognition of pregnancy in the sheep (Godkin *et al.*, 1984a). If so it seemed likely that they would either enter the maternal system and act on the ovaries directly, or alternatively, assert their action *via* the endometrium. The latter alternative was considered more likely since conceptus homogenates only prolong the cycle if they are introduced into the uterine lumen.

To test whether secreted conceptus protein could extend CL function Godkin *et al.* (1984b) infused proteins released by day 15–16 conceptuses into the uterine lumen of cyclic ewes. Beginning on day 12 either a concentrated solution of total secreted proteins or diluted sheep serum was introduced daily *via* an indwelling catheter into the uterine lumen of 3 ewes for 7 days (days 12–18). Peripheral blood samples were collected daily for 14 days (days 12–25). On day 25, all ewes were laparotomized and ovaries observed to determine whether CL, previously marked with India ink, were maintained. All controls had ovulated and formed new CL. By contrast, none of the ewes treated with conceptus proteins had ovulated, and their peripheral progesterone levels remained elevated. One ewe maintained a functional CL until day 52, when she was hysterectomized. Light microscopy of histological sections prepared from the endometrium revealed glandular development comparable to endometrium of day 60 pregnant animals. The cells of the CL were similar to those from cyclic animals during mid to late diestrus.

oTP-1 was also introduced into the uterine lumen of 3 animals. In these, plasma progesterone concentrations were maintained for an average of 4 days longer than in control animals (Godkin *et al.*, 1984b).

As a result of the above experiments we suggest that secreted proteins of the conceptus are involved in the maintenance of luteal function during early pregnancy. It is not clear why oTP-1 was not as effective as total secreted conceptus protein in prolonging the cycle. Possibly it was introduced at too low a concentration to provoke a

complete and effective response from the maternal system. Moreover, oTP-1 may have been partly degraded during purification. Another possibility is that because we applied only small amounts of pure oTP-1 it was not protected adequately from proteolytic destruction within the uterus. The infusion of greater quantities of oTP-1 or the addition of protein carrier might reduce the amount of oTP-1 degradation and allow greater quantity of active peptide to be available for interaction with maternal tissues. Finally, it is conceivable that oTP-1 constitutes just one component of an antiluteolytic complex, and that other conceptus proteins make up the remainder of this complex.

Some initial experiments have also been conducted to determine how oTP-1 interacts with the maternal system. Using a specific antiserum raised against oTP-1, Godkin *et al.* (1984a) demonstrated that the protein was associated with trophectoderm cells of the elongating blastocyst and with the surface and upper glandular epithelium of the maternal uterus. Receptors which bound oTP-1 with high affinity ( $k_d = 2 \times 10^{-10}$  M) were present in crude membrane fractions prepared from homogenates of endometrium. Uterine infusion of [ $^{125}$ I]-labelled oTP-1 into day 12 nonpregnant ewes showed that the majority of the radioactivity was retained in the uterus and did not enter the maternal vasculature. There was no significant association with the CL, ovaries or other tissues tested. oTP-1 failed to compete with ovine prolactin for rabbit mammary receptors or with human chorionic gonadotrophin or bovine luteinizing hormone for sheep luteal cell receptors; nor did oTP-1 stimulate progesterone production by dispersed luteal cells from day 12 cycling ewes. Incubation of endometrial explants from day 12 nonpregnant ewes with 5  $\mu$ g/ml oTP-1 resulted in increased rates of release of newly synthesized protein into the medium. Two-dimensional polyacrylamide gel electrophoresis revealed that the synthesis of at least 6 polypeptides was stimulated selectively by the presence of oTP-1.

The above data suggest that oTP-1 acts on the maternal endometrium and not directly on the CL or other extrauterine tissues. It is suggested that these biochemical interactions with the endometrium may elicit maternal responses which contribute to the maintenance of pregnancy in the sheep.

**Cow:** Maternal recognition of pregnancy in the cow is believed to occur around day 16. Intrauterine injections of embryonic homogenates (17 to 18 days of age) during days 15 to 17 of the estrous cycle extended the interestrus interval to 24 days from 21.1 days for controls and delayed the decline in progesterone associated with CL regression (Northey and French, 1980). Bartol *et al.* (1982) have shown that cow conceptuses isolated during this critical period of pregnancy secrete two major polypeptide products. As with the sheep and pig, one was a high molecular weight glycoprotein (Masters *et al.*, 1983). The other was of much lower molecular weight ( $\sim 20,000$ – $25,000$ ) and fairly acidic in nature. Like the similar sheep protein it consisted of several isoelectric species. Unlike oTP-1, however, this protein has not yet been purified. Nevertheless the introduction of total secreted proteins from cow conceptuses into day 16 nonpregnant cows led to an average nine day extension of their estrous cycles (Knickerbocker *et al.*, 1984). It seems possible, therefore, that the mechanisms for maternal recognition of pregnancy in the cow and the sheep may be similar, and that the chemical signal is a protein secreted by the early elongating blastocyst.

## Concluding remarks

It has become clear that although the elongating blastocysts of the pig, sheep and cow depend upon an appropriate uterine milieu for their growth and development, they are not passing occupants but are involved in controlling this intrauterine environment by generating chemical signals which cause appropriate responses in the maternal system. These signals may include the production of steroid hormones and the release of secreted proteins which presumably also have a hormone-like function. Available evidence suggests that this type of endocrine signaling from the conceptus begins at about the time the blastocyst begins to elongate from a sphere to a long thread-like form.

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## Effect of super-ovulatory doses of pregnant mare serum gonadotropin and human chorionic gonadotropin in blastocyst implantation in golden hamsters

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**Abstract.** The effect of super-ovulatory dose of pregnant mare serum gonadotropin and human chorionic gonadotropin on ovulation, advancement of ovulation, subsequent embryo development and implantation were studied in the hamster. Groups of hamsters received pregnant mare serum gonadotropin injection on day 1 of the estrous cycle followed by human chorionic gonadotropin injection either at 56 or 76 h later, pregnant mare serum gonadotropin alone on day 1 or human chorionic gonadotropin alone on day 3.

The combination therapy (pregnant mare serum gonadotropin and human chorionic gonadotropin) resulted in super-ovulation (an average of 40 mature ova/animal) while human chorionic gonadotropin alone yielded an average of 10 mature ova/animal. Ovulation was advanced by 24 h by giving human chorionic gonadotropin at 56 h instead of 76 h after pregnant mare serum gonadotropin. Subsequent embryo development and implantation occurring under different hormonal regimens were studied. The ova obtained by giving human chorionic gonadotropin injection at 56 h were poorly fertilizable *in vivo* and hence the pregnancy rate was low (6%). These ova however, were fertilizable *in vitro*, suggesting that the low fertilization rate and developmental failure may be due to inhibition of sperm capacitation/transport because of premature human chorionic gonadotropin administration. In the group receiving human chorionic gonadotropin alone on day 3 there was fertilization and cleavage, but no implantation occurred due to failure of functional corpora lutea. However, administration of progesterone and estrone from day 2 of gestation resulted in 80% implantation and sustenance of pregnancy. On the other hand, the pregnant mare serum gonadotropin and human chorionic gonadotropin combination therapy resulted in super-pregnancy. The number of fetuses present at term was higher in the group receiving pregnant mare serum gonadotropin alone than in the group receiving the combination therapy. Embryo resorption however was higher (37%) in the latter group compared with the former (9.5%). However, preimplantation embryos were found to be viable as evidenced by fluorescein diacetate staining.

**Keywords.** Gonadotropins; embryo viability; implantation.

### Introduction

In contrast to the normal process of ovulation, induction of ovulation by exogenous administration of hormones results in a large number of eggs being released. However, it is questionable whether all these eggs are fertilizable *in vivo* and whether subsequent embryo development can continue till term.

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Abbreviations used: PMSG, Pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin, HSA, human serum albumin; PBS, phosphate buffered saline, 0.01 M, pH 7.4, 0.9 NaCl, normal saline; FDA, fluorescein diacetate.

In the present paper we have evaluated a number of biological parameters in hamsters exogenously treated with the hormones, pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The parameters studied were ovum maturity, *in vivo* fertilization, normality and viability of embryos, ability of the blastocysts to implant and development of the fetuses to term.

### Materials and methods

Golden hamsters were maintained under a light:dark schedule of 14:10 h. Females 2 to 4 months old showing at least two consecutive estrous cycles were mated with 4–6 months old male breeders. The day of the post estrous vaginal discharge was designated as day 1 of cycle.

Four different hormone regimens were used. Females in group 1, 2 and 3 received 30 I.U. of PMSG (NIH) on day 1 of the estrous cycle at 10:00 h. Those in group 1 received 30 I.U. of hCG (Sigma) on day 4 (76 h after the PMSG injection). While those in group 3 received 30 I.U. hCG on day 3 (56 h after the PMSG injection). Females in group 2 were not administered hCG. Females in group 4 did not receive PMSG but received 30 I.U. hCG on day 3 at 15:00 h. Ovulation was advanced by 24 h in groups 3 and 4.

In the first experiment the maturity of the ova was assessed. Females in group 1, 3 and 4 were sacrificed 16–18 h after the hCG injection; those in group 2 were sacrificed between 08:00 and 10:00 h on the day of ovulation. The oviducts were dissected out and the cumulus masses were flushed with BWW medium (Biggers *et al.*, 1971), containing 0.3% human serum albumin (HSA-Sigma) using a number 30 gauge blunt needle inserted through the fimbrial end. The cumulus cells were dispersed using 0.1% hyaluronidase solution (Sigma). After removal of the cumulus, the ova were counted and checked for their maturity.

In the second experiment, the normality and viability of the superovulated embryos was studied. Females in groups 1 and 2 were mated overnight from the evening of day 4 of cycle. Females in group 3 and 4 were mated overnight from the evening of day 3 of cycle. Vaginal smears were taken the following morning to check for mating. This day was considered as day 1 of pregnancy.

On day 3 of pregnancy, between 17:00 and 19:00 h the animals were sacrificed and the oviducts were flushed with 0.01 M phosphate buffered saline (PBS) pH 7.4 containing 0.4% bovine serum albumin (Sigma). Embryos were assessed for normality by their morphology and expected developmental stage. Viability of embryos obtained in group 1 and 2 was assessed by fluorescein diacetate (FDA) (Sigma) staining. Embryos were incubated in FDA solution for approximately one minute at room temperature (27–33°C). The concentration of FDA used was 2.5 µg per ml PBS. Viable embryos fluoresce brightly while nonviable ones show absence of fluorescence or markedly diminished fluorescence.

In the third experiment, mated females from each group were sacrificed on day 8 or day 14 of pregnancy. The number of implantation sites or fetuses as well as the number of resorption sites or unilateral pregnancies were recorded.

In the fourth experiment exogenous progesterone was administered to animals in

group 4 at different dose levels according to the protocol of Sehgal and Diamond (1977) soon after mating. Additionally 2 other regimens were used. The first comprised of daily administration of 2 mg of progesterone from day 2 of pregnancy. The latter was a combination therapy of 2 mg progesterone and 1  $\mu$ g estrone (Sigma).

## Results

In groups 1, 2 and 3 an average of 40 mature ova (as evidenced by release of the 1st polar body) were obtained per animal. An average of 10 mature ova per animal was obtained in group 4.

Animals belonging to groups 1, 2, 3 were sacrificed on day 3 of pregnancy. This was the optimal timing for obtaining embryos at 8 cell stage. The embryos and unfertilized ova (if present) obtained in groups 1 and 2 were ascertained for viability using FDA. The results of FDA staining are indicated in table 1. All embryos at the expected developmental stage (*i.e.* 8 cell stage) were viable, as they fluoresced brightly when stained with FDA (figure 1). Unfertilized ova were all non-viable. The number of embryos at 8 cell stage was 75% in group 1 and 86.5% in group 2. In group 3, very few

Table 1. Effect of PMSG and hCG treatment on embryo viability.

Animal group	Hormone administration	Total No. of embryos/ova examined	Developmental stage	FDA test		
				No. positive	No. partial	No. negative
1	PMSG day 1	146	8 cell	110	—	—
	hCG day 4	—	2-6 cell	—	—	—
2	PMSG day 1	164	8 cell	142	—	—
			3-4 cell	12	—	—
			2 cell	—	6	—
			Unfertilized	—	—	4



A



B

Figure 1. A. Hamster embryo at 8 cell stage (phase contrast). B. Same embryo brightly fluorescent when stained with FDA.

ova were obtained after flushing. All of them were unfertilized and were degenerating (figure 2).

Animals in group 4 were sacrificed at different times following fertilization and their developmental stages were noted (table 2). Fertilization occurred in this group. However, 22 to 32% of the embryos were abnormal as compared to 5% abnormal embryos obtained in the control group.

Animals in groups 1 and 2, autopsied on day 8 of pregnancy were found to be superpregnant (table 3). The pregnancy rates in the two groups were 88% and 87% respectively. The mean number of implantations per pregnant female was 26 in group 1 and 23 in group 2. However, in both groups there was a reduction in the average number of fetuses obtained on day 14 of pregnancy. It was 11.3 in group 1 and 19.8 in group 2. The reduction was the result of resorption. Resorption sites were also observed in some animals autopsied on day 8. The percentage of animals showing resorption was higher in group 1 (37%) as compared to group 2 (9.5%). Six per cent of the pregnancies were unilateral in group 1 and 9.5% in group 2.

In group 3, the pregnancy rate was very low (6%) (table 3). One pregnant animal

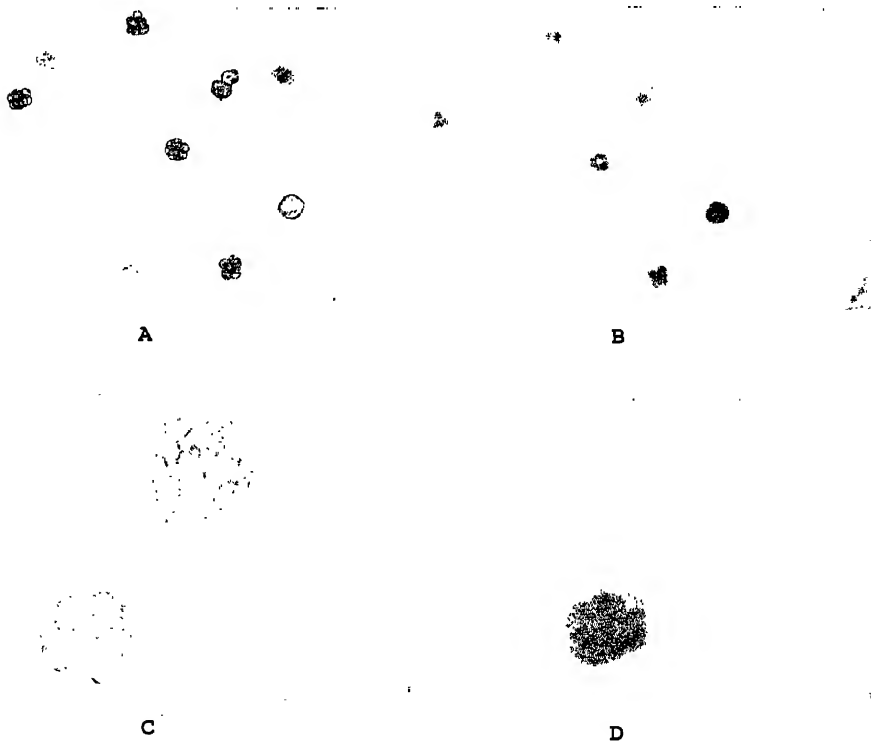


Figure 2. A. Embryos and unfertilized eggs (arrows) at low power (phase contrast). B. FDA staining; note absence of fluorescence of the degenerating (unfertilized) eggs, while the other embryos are fluorescent. C and D. Phase contrast and fluorescence of an 8 cell embryo and an unfertilized egg.

Table 2. Effect of hCG administration on day 3 on embryo development.

Animal group	No. of females mated	Time when embryos were collected (estimated hours after fertilization)	Site of embryos	Developmental stage of embryos	Embryos collected		
					Total no.	No. (%) normal	No. (%) abnormal
Control*	3	5-11	Oviduct	Pronuclei	33	33 (100)	—
	3	27-31	Oviduct	2 cell	32	32 (100)	—
	7	51-63	Oviduct	4 to 8 cell	71	71 (100)	—
	5	64-70	and uterus Uterus	8 to blastocyst	38	36 (95)	2 (5)
Experiment**	3	5-11	Oviduct	Pronuclei	27	27 (100)	—
	7	27-31	Oviduct	2 cell	71	68 (96)	3 (4)
	14	51-63	Oviduct	4 to 8 cell	73	50 (68)	23 (32)
	4	64-70	and uterus Oviduct and uterus	Blastocyst	9	7 (78)	2 (22)

\* No hormone administered. Animals mated on evening of day 4 of cycle.

\*\* 30 I.U. hCG administered on day 3. Animals mated on day 3 evening.

Table 3. Effect of PMSG and hCG treatment in super-pregnancy.

Animal group	Hormone administration	No. of females mated	Day of examination	No. of animals examined	Mean No. ( $\pm$ S.E.M) of implantations/fetuses per pregnant female
1	PMSG day 1	16/18	8	4*	26 $\pm$ 1.87
	hCG day 4	(88)**	14	9	11.3 $\pm$ 2.02
2	PMSG day 1	21/24	8	12	23 $\pm$ 2.48
	Mated day 4	(87)**			19.8 $\pm$ 2.97
3	PMSG day 1	2/32	8	1	11
	hCG day 3	(6)**	14	1	16
	Mated day 3				

\* Complete resorption in the other three animals.

\*\* No. in parenthesis indicate % pregnancies obtained.

showed 11 implantations on day 8, while another sacrificed on day 14 showed 16 fetuses. In group 4 implantation did not occur.

Exogenous progesterone at a dose of 2 mg when administered daily, starting from day 2 of pregnancy resulted in implantation, but pregnancy was not sustained (table 4). Similar results were obtained when single injections of 5 mg and 10 mg of progesterone (Sehgal and Diamond, 1977) were administered soon after mating. However, simultaneous administration of estrone (1  $\mu$ g) and progesterone (2 mg) daily, commencing from day 2 of pregnancy resulted in 80% implantation and pregnancy was sustained to term.

Table 4. Effect of steroid administration on implantation and sustained pregnancy.

Animal group	hCG administration	No. of animals	Hormone treatment (following mating)	Day of examination	Results
4	30 IU. hCG on day 3 at 15 h	5	2 mg progesterone (oil). Daily subcutaneous, starting from 37 h. After mating (2-cell stage) After artificial insemination	Day 14	4 Females ... No sign of implantation 1 Female ... 5 normal size embryos and 2 resorption sites 1 implantation site
	—do—	1		Day 8	
	—do—	6	5 mg progesterone (oil) single subcutaneous soon after mating	Day 14	3 Females ... no sign of implantation- 1 Female ... 7 Resorption 1 Female ... 13 Resorption 1 Female ... 5 Resorption
	—do—	2	10 mg progesterone (oil) single subcutaneous soon after artificial insemination	Day 14	1 Female ... no sign of implantation
		1	10 mg progesterone (oil)	Day 14	No sign of implantation
		5	1 µg estrone/2 mg progesterone (subcutaneous daily 10 a.m., starting from 37 h after mating)	Day 8	1 Female ... no sign of implantation 1 Female ... 9 embryos 1 Female ... 10 embryos 1 Female ... 8 embryos 1 Female ... 8 fetuses
				Day 14	

## ssion

Results of this study establish the maturity of the ova obtained with all 4 hormone regimens used. Super-ovulated preimplantation embryos obtained using hormone regimens 1 and 2 were viable as evidenced by FDA staining. FDA is a non-polar compound and hence can readily cross the cell membrane. Once within the cell, it is hydrolysed by esterases to yield fluorescein. The latter being polar cannot pass out of the cell. Thus, the presence of fluorescence within the blastomeres indicates both metabolic activity which is present only in living cells (Rotman and Papermaster, 1966), as well as membrane integrity. This technique is useful because the stained embryos can be transferred back to the uterus and they are not affected by the staining.

There was a decline from the average number of embryos present on day 8 of pregnancy to that obtained on day 14 of pregnancy in groups 1 and 2. The resorption of embryos is probably due to the inability of the uterus to accommodate a large number of embryos to term. At present it is not clear if the higher resorption rate in group 1, is due to the presence of a hostile environment in their uteri. Perhaps transfer of viable 8 cell embryos from groups 1 and 2 to synchronized albino recipients, may provide an answer.

The unilateral pregnancies which occurred in groups 1 and 2 may be due to asynchrony between embryo development and uterine receptivity to implantation.

The average number of fetuses obtained on day 14 was 11.3 in group 2. This figure is lower than that reported by Fleming and Yanagimachi (1980). In group 2, the average number of implantations was 23 which is lower than the average number of 29 implantations reported by Greenwald (1979). However, the number of fetuses present on day 14 was 19.8 which is comparable to the value of 20.8 fetuses reported by Greenwald.

Hormone regimen 3 resulted in low rates of fertilization and sustained pregnancy. Super-ovulated ova obtained by this hormone regimen are fertilizable *in vitro* (Yanagimachi and Chang, 1964). Therefore the causes for developmental failure probably include failure of sperm transport or capacitation due to hCG administration by regimen 3 (Chang 1970). Endocrine disturbances in the maternal or fetal environment may also have resulted due to elevated steroid concentrations. Artificial insemination may be carried out to ascertain if the fertilization rate increases.

In hormone regimen 4, 68–78% of the preimplantation embryos were normal but all failed to implant, perhaps due to failure of corpus luteum function. Only administration of progesterone and estrone from day 2 to day 14 of pregnancy sustained pregnancy to term. This finding is surprising since implantation in the hamster has been reported to occur with progesterone alone (Orsini and Psychoyos

In the four hormone regimens used, the results of administration of hCG alone on pregnancy are of interest. Advancement of ovulation does not seem to affect fertilization, but there is a higher percentage of abnormal cleavage compared to the control. Further, implantation is prevented due to failure of corpus luteum function. The results of the transfer experiment in all these groups perhaps, would provide the reason for the observation. This would help in understanding the effect of the donor's uterine environment to which the embryos have been subjected, on further development.



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## ***In vitro* fertilization and preimplantation development in the primate**

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**Abstract.** Nonhuman primates represent a strong model for examining the chromosomal, biochemical, and temporal normality of embryos produced by *in vitro* fertilization. More *in vitro* fertilized embryos from the squirrel monkey (*Saimiri sciureus*) have been produced and examined than with all other primate species combined. In studies over a 13 year period a fertilization rate approximating 60% has been developed in this species with 30% of these embryos proceeding to the two cell stage and 50% of these to the three-four cell stage. Chromosomal abnormalities (primarily missing or extra chromosomes) at a level of nine to 16% have been found, a value corresponding to that found in *in vivo* mating and *in vitro* fertilization in other species. An incidence of triploidy of 16.7% was observed. RNA and protein synthetic rates appear comparable with those of laboratory species subjected to *in vitro* fertilization and indicate the initial stages of metabolic activity of the newly formed embryo. Similarly, increases in estrogen incorporation appear after fertilization but no effect is observed in progesterone incorporation. Utilizing 2-deoxy-glucose and insulin, it was determined that the glucose requirement as an energy source for early preimplantation *in vitro* fertilized primate embryos is very low.

Of very great importance is the temporal relationship of the development of *in vitro* fertilized squirrel monkey embryos compared with similar development in other primates (including humans) after *in vivo* and *in vitro* fertilization. An analysis of over a decade of work with the squirrel monkey embryos demonstrates a pattern of temporal development that is comparable with all other primate species that have been examined (including the human) and comparable with development after *in vivo* fertilization.

**Keywords.** Fertilization; implantation; primate; embryos.

### **Introduction**

*In vitro* fertilization in laboratory animals was first achieved in the 1950's, but it required over 20 years before this technique was successful with humans. Since the first human birth in 1978, a large number of *in vitro* fertilization clinics have developed around the world and several hundred pregnancies have been established. Because of ethical and moral constraints it is impossible to subject *in vitro* fertilized human embryos to many of the common cytogenetic and biochemical assays to determine normality. While none of the children born of this procedure have yet shown abnormal types of development, it is desirable that scientific studies be undertaken, using nonhuman primates as a model, on the development of embryos produced by *in vitro* fertilization.

Several research groups have reported successful *in vitro* fertilization in the nonhuman primate. The efforts of our laboratory were first fully reported in 1975 (Dukelow and Kuehl, 1975; Kuehl and Dukelow, 1975a). Since that time a number of

publications have appeared from our laboratory relating to *in vitro* fertilized primate oocytes and were recently summarized (Dukelow *et al.*, 1983).

To successfully achieve a pregnancy it is necessary to not only *in vitro* fertilize the oocyte but also to effectively transfer the embryo to a recipient female. The first surgical transfer of an *in vivo* fertilized embryo was accomplished in the baboon by Kraemer *et al.* (1976). The first nonsurgical transfer was carried out by Pope *et al.* (1983) in the baboon. This involved an *in vivo* fertilized embryo. Finally, on July 25, 1983, exactly five years after the birth of the first human *in vitro* fertilized embryo transfer baby, a baboon was born by this procedure (Kuehl *et al.*, 1983). At the time of the writing of this manuscript, another baboon is pregnant as a result of transfer of an *in vivo* fertilized embryo that had been frozen, stored, and thawed (Dr. C. E. Pope, personal communication).

Thus it would appear that all of the procedures necessary for examining the normality of nonhuman primate offspring resulting from *in vitro* fertilization are available and tested. In addition to the research groups already cited, others have reported preliminary studies indicating successful *in vitro* fertilization in the rhesus monkey (Bavister *et al.*, 1983), the cynomolgus macaque (Kreitmann *et al.*, 1982) and the chimpanzee (Gould, 1983).

## Materials and methods

All of the present studies were carried out with adult squirrel monkeys (*Saimiri sciureus*) of Brazilian or Guyanan origin. During the winter months the animals were housed in stainless steel, flush type cages which conformed to state and federal regulations. During the summer months the animals were housed out-of-doors in a cage system previously described by Jarosz and Dukelow (1976). The colony was composed of 140 females and 9 males. They received a commercial monkey diet with supplemental apples. Fresh water was supplied *ad libitum*. The animal room had fluorescent lighting on a 12L:12D cycle and was temperature controlled at  $21 \pm 2^\circ\text{C}$ .

Because the estrous cycle of a squirrel monkey is not evident (there is no menstruation and vaginal cytology is not precise enough to predict ovulation), follicular development was induced in the animals by a regimen of four days of follicle stimulating hormone (FSH) (1 mg per day) followed by a single injection of 250 IU of human chorionic gonadotropin (hCG, APL,® Ayerst Company, Montreal, Canada). This regimen was previously published (Dukelow, 1970) and has been used in our laboratory for 15 years. During the summer months, because of the seasonal responsiveness of the squirrel monkey (Harrison and Dukelow, 1973) the period of FSH injection was extended to five days (Kuehl and Dukelow, 1975b). The minimum effective dose of hCG to induce ovulation has previously been determined to lie between 100 and 250 IU hCG (Dukelow, 1979). Semen was collected by the restraint and ejaculation procedure previously described (Kuehl and Dukelow, 1974). A coagulated ejaculate was incubated 30 min at  $37^\circ\text{C}$  and the resulting sperm suspension used to inseminate oocyte cultures.

Oocytes were recovered 16 h after the hCG injection. This was accomplished laparoscopically (Dukelow and Ariga, 1976). The oocytes were aspirated into 0.1 ml of culture media and allowed to mature for 21 h prior to the addition of sperm. The

culture media used was TC 199 supplemented with 1 mmol pyruvate, 100 mg/ml of gentamicin and 1 IU/ml heparin. Twenty per cent fetal calf serum that had been heat-inactivated for 30 min at 56°C was added.

Oocyte maturation *in vitro* follows a linear pattern for about 40 h after recovery. By 21 h of culture about 50% of the oocytes are matured (Chan *et al.*, 1982).

The recovered oocytes with associated cumulus cells, follicular fluid and media were placed in tissue culture chamber slides. These were incubated at 37°C in a moist atmosphere of 5% CO<sub>2</sub> in air and checked at intervals for evidence of fertilization.

To assess the chromosomal normality of oocytes and embryos produced by *in vitro* fertilization, a final concentration of 0.04 gm/ml of colchicine was dissolved in some culture chambers 12 h after insemination. Oocytes were examined for the presence of polar bodies after removal of the cumulus cells. The oocytes or embryos were then prepared for chromosome analysis by a gradual fixation method with moist air (Mizoguchi and Dukelow, 1981). The oocytes were incubated in 0.1% trypsin in TC199 medium for 5 min to soften the zonae pellucidae. For hypotonic treatment the oocytes were incubated in 35% newborn calf serum for 30 min at 37°C. All preparations of oocytes and embryos were stained with 2% giemsa.

To assess the RNA synthetic rate, oocytes or embryos were incubated for three hours in modified TC199 medium with 20% fetal calf serum supplemented with 5-[<sup>3</sup>H]-uridine (final concentration 2.8 μM; specific activity, 18 Ci/mmol). This concentration allowed maximum incorporation to take place. The embryos were washed 10 times in nonradioactive medium, solubilized, and trichloroacetic acid-precipitable and non-precipitable material counted. Additionally, some embryos were fixed in Bouin's fluid after incubation with [<sup>3</sup>H]-uridine and processed for autoradiography. The relative amount of uridine incorporation into RNA was determined by counting the reduced silver grains over a 300 μM<sup>2</sup> area of nucleoplasm or cytoplasm. These counts were converted to 1000 μM<sup>2</sup> for ease in calculation and graphic representation.

To assess the protein synthesis, autoradiographic procedures were used with the culture media supplemented with 0.4 μM L-4, 5-[<sup>3</sup>H]-leucine (specific activity, 50.4 Ci/mmol). Incubation took place for 3 h. The oocytes or embryos were then washed ten times with a medium containing unlabelled leucine, fixed 24 h in Bouin's fluid and processed for autoradiography. In addition, the ova were incubated for 2 h in 0.2 ml of culture medium with 0.06 μM of either labelled estradiol or progesterone. Ova were washed ten times in phosphate buffers, dissolved in 0.1 ml of tissue solubilizer, and the solutions assayed for radioactivity.

Additionally studies with 2-deoxy-D-glucose (2-DG) were carried out to assess the effects of insulin addition to the media and assess the importance of glucose as an energy source to early preimplantation primate embryos. Oocytes or *in vitro* fertilized embryos were preincubated for 1 h in 0.2 ml of media containing 5.56 mM D-glucose and washed five times. They then were incubated in media containing 2-deoxy-D-1-[<sup>3</sup>H]-glucose (specific activity, 25 Ci/mmol). Accumulation over a 3 h culture period was measured with and without the addition of 10 nM or 1 μM insulin from bovine pancreas. Following incubation the embryos or oocytes were washed ten times, solubilized, and radioactivity measured.

Finally, the rate of development of embryos in *in vitro* culture was determined (from 11 collected data over a ten year period) to provide a temporal scale of development.

This was compared with known values for both *in vitro* and *in vivo* fertilized embryos in the rhesus monkey, cynomolgus monkey, baboon and human.

## Results

In the most recent summary, 995 oocytes have been recovered laparoscopically from the squirrel monkey. Of these 628 (31.5 %) matured and 339 (54.0 %) fertilized *in vitro*. In studies completed in 1981 and 1982 the *in vitro* fertilization rate approximated 60 % and 30 % of these developed *in vitro* to the two cell stage. Of these 52 % advanced to the three-four cell stage and embryos have been developed to the 16 cell stage. The latter have been subjected to electron microscopic evaluation (Yorozu *et al.*, 1984). Electron-microscopic analysis indicates normality of the embryos.

Examining nonfertilized oocytes for chromosomal normality 666 oocytes were recovered from 2150 follicles. Of those oocytes assessed at the metaphase II stage from 7.4 to 14.0 % exhibited abnormalities, normally missing or extra chromosomes. This value is comparable to that found in other laboratory species. Similar studies were carried out with oocytes exposed to *in vitro* fertilization conditions. Results are shown in table 1. Again, the incidence of abnormalities ranged from nine to 16 % with the common abnormalities being missing or extra chromosomes. These values are also comparable to that found with natural fertilization and *in vitro* fertilization in a variety of laboratory species. Of interest was a relatively high incidence of triploidy of 16.7 %. Triploidy has been reported to be a common problem with *in vitro* fertilization systems in other species including the human.

Table 1. Chromosomal abnormality incidence in *in vitro* fertilized squirrel monkey oocytes.

Developmental stage	Incidence of abnormality
Metaphase I	3/18 (16.7 %)
Anaphase I	0/5
Metaphase II	
Haploidy	5/72 (8.9 %)
Polarbody	4/29 (13.8 %)
First cleavage metaphase	
Aneuploidy	3/25 (12.0 %)
Triploidy	5/30 (16.7 %)

RNA synthesis rate as measured by [ $^3\text{H}$ ]-uridine incorporation is shown in table 2. Autoradiographic analysis demonstrated a significant increase in RNA synthetic capacity, after *in vitro* fertilization and again after the second cleavage division had begun. [ $^3\text{H}$ ]-Leucine incorporation by a squirrel monkey oocyte decreased after a 21 h maturation period. The grain counts remained the same after *in vitro* fertilization with a nonsignificant elevation at the first cleavage (table 3).

Steroid uptake was analyzed for embryos fertilized *in vitro* and there was a trend for increased estradiol uptake with *in vitro* fertilization and first cleavage (table 4) although

**Table 2.** [ $^3\text{H}$ ]-Uridine incorporation by *in vitro* fertilized squirrel monkey ova.

Cell stage	<i>n</i>	No. of grains/1000 $\mu\text{M}^2$ <sup>a</sup>
Unfertilized	8	25 $\pm$ 3
Fertilized	11	39 $\pm$ 3 <sup>b</sup>
Two-cell	10	44 $\pm$ 4
Three-cell	1	75 <sup>b</sup>

<sup>a</sup> Number of reduced silver grains per 1000  $\mu\text{M}^2$  of embryonic nucleoplasm or ooplasm  $\pm$  standard error.

<sup>b</sup> Significantly different from previous cell stage ( $P < 0.05$ ).

**Table 3.** [ $^3\text{H}$ ]-Leucine incorporation by squirrel monkey oocytes and by embryos produced by *in vitro* fertilization.

Cell stage	<i>n</i>	No. of grains/1000 $\mu\text{M}^2$
Immature oocyte	10	329 $\pm$ 20
Mature oocyte	8	164 $\pm$ 20 <sup>a</sup>
Fertilized	7	118 $\pm$ 40
Two-cell	3	220 $\pm$ 25

<sup>a</sup> Significantly different from previous cell stage ( $P < 0.05$ ).

**Table 4.** [ $^3\text{H}$ ]-Estradiol and [ $^3\text{H}$ ]-progesterone uptake by squirrel monkey oocytes and by embryos produced by *in vitro* fertilization.

Cell stage	<i>n</i>	Uptake (pmol/embryo/2h)	
		Estradiol	Progesterone
Unfertilized	8	0.59 $\pm$ 0.07	0.21 $\pm$ 0.02
Fertilized	5	0.87 $\pm$ 0.17	0.49 $\pm$ 0.05
Two-cell	4	1.20 $\pm$ 0.40	0.38 $\pm$ 0.10

this was not statistically significant. Progesterone uptake increased at *in vitro* fertilization and then remained constant at the two cell stage. The results with 2 DG have been reported elsewhere (Hutz *et al.*, 1984). Briefly summarized, the addition of insulin increased 2-DG accumulation by unfertilized oocytes over controls without insulin, but not to a significant extent (from 13.95  $\pm$  2.4 to 15.64  $\pm$  3.78 to 18.84  $\pm$  1.22 fmol/oocyte/3 h) for the control and two levels of insulin respectively. There was no change in 2-DG accumulation at fertilization. These results suggest low utilization of glucose by the early preimplantation primate embryos similar to that demonstrated

for other mammalian species. 2-DG did appear to be a good viability indicator of early primate embryos.

The temporal stages of development for squirrel monkey oocytes in the preliminary stages of development have already been reported (Dukelow *et al.*, 1983). Capacitation in this species requires 2-4.7 h (Kuehl and Dukelow, 1982) and by 6 h after fertilization condensation and swelling of the sperm head has occurred. The first pronucleate stage is evident by 10 h after fertilization with initial cleavage occurring from 16 to 20 h. Subsequent development of the squirrel monkey embryo, compared with the rhesus monkey, cynomolgus monkey, baboon and human are indicated in table 5.

Table 5. Comparative rates of primate preimplantation development.

Species (type of culture)	Two polar bodies	(Hours after fertilization)					
		2 Cell	4 Cell	8 Cell	16 Cell	Morulla	Blastocyst
Squirrel Monkey							
<i>in vivo</i> fert., <i>in vivo</i> culture <sup>a</sup>							96
<i>in vitro</i> fert., <i>in vitro</i> culture <sup>b</sup>	6-22	20-40	46-52	52-72			
Rhesus Macaque							
<i>in vivo</i> fert., <i>in vitro</i> culture <sup>c</sup>		24-36	36-48	48-72	72-96		
<i>in vitro</i> fert. <i>in vitro</i> culture <sup>d</sup>		26	38	54	87		
Cynomolgus Macaque							
<i>in vivo</i> fert., <i>in vitro</i> culture <sup>e</sup>		24	48	48-72			
Baboon							
<i>in vivo</i> fert., <i>in vivo</i> culture <sup>f,g</sup>	24		48	48-72	96-120	120-148	96-144
<i>in vitro</i> fert., <i>in vitro</i> culture <sup>h</sup>	6-12	24		72*			
Human							
<i>in vivo</i> fert., <i>in vivo</i> culture <sup>i,j,k</sup>		30		72	96	96-120	120
<i>in vitro</i> fert., <i>in vitro</i> culture <sup>l</sup>	12	38	38-46	51-62	85	111-135	123-147

\* Xenogenously fertilized. a) Ariga and Dukelow, 1977; b) Kuehl and Dukelow, 1979; c) Lewis and Hartman, 1941; d) Bavister *et al.*, 1983; e) Kreitmann *et al.*, 1982; f) Kraemer and Hendrickx, 1971; g) Pope *et al.*, 1982; h) Kuehl *et al.*, 1984; i) Hertig, 1975; j) Croxatto *et al.*, 1972; k) Avendano *et al.*, 1975; l) Edwards and Steptoe, 1975.

## Discussion

Based on the recovery and fertilization rate of squirrel monkey oocytes, this animal provides a very useful model for the examination of other facets of abnormality. With

increasing use of *in vitro* fertilization in human infertility cases there remains the problem of genetic safety of *in vitro* maturation and fertilization procedures. In the present studies, hormonal induction of follicular development and *in vitro* fertilization were found not to be major causes of chromosomal abnormality. The levels of missing or extra chromosomes were found to be quite comparable to that reported for other laboratory species. The single measure that was adverse to the *in vitro* fertilization system was the 16.7% incidence of triploidy, probably caused by polyspermy. The exact cause of this polyspermy is unknown at this time, but a similar event has been noted in the *in vitro* fertilization system of other laboratory animals and humans (Lopata *et al.*, 1978). Varying numbers of sperm were utilized in the culture system to yield the present data and presently a comprehensive analysis of the effect of sperm number on the incidence of polyspermy is being undertaken.

Autoradiographic analysis of RNA synthetic capacity after *in vitro* fertilization and cleavage showed that uridine uptake is many times lower than similar studies with hamster embryos (Hutz, 1983). Precursor pools may differ appreciably between species. Nevertheless, RNA synthesis did appear to remain quite low in early preimplantation development and this correlates well with the very low RNA synthesis found in the early preimplantation stages of the mouse (Knowland and Graham, 1972). In that species, increases of RNA synthesis are not seen until the eight cell and morula stages and again at the blastocyst stage. Similarly the decrease of the cellular incorporation of leucine with maturation and the low levels at fertilization are similar to that found in other mammalian species (Baker *et al.*, 1969; Brinster, 1971; Schultz *et al.*, 1978). These low levels of protein synthesis correlate with the low number of polyribosomes found in the morula and blastocyst stages of primate embryos fertilized *in vivo* (Enders and Schlafke, 1981) and *in vitro* (Yorozu *et al.*, 1984). Since the size of the precursor pool for leucine is not known for primate ova, absolute measurements of protein synthesis could not be made. Apparent increases in protein synthesis occur late in preimplantation development (at the eight cell and blastocyst stages) in the mouse (Epstein, 1975).

The studies demonstrating uptake of estradiol by primate oocytes after fertilization apparently indicate that steroid receptors are present very early in the development but their functional significance is unknown. Because of the scarcity of available material we have not been able to separate cytosol from nuclear receptors in primate ova. Steroids can effect various aspects of metabolism but do not alter RNA synthetic rates in other laboratory animals. Steroids may rather play a role in effecting membrane changes and therefore cleavage of the early preimplantation embryos (Warner and Tollefson, 1978). Near the time of implantation embryos possess aromatase activity and are able to synthesize steroids (Dickmann and Dey, 1974; Hoversland *et al.*, 1982).

These biochemical studies demonstrate normal development of preimplantation primate embryos, similar to that of other mammalian species, after *in vitro* fertilization in culture. Such investigations allow evaluation of the requirements of the embryo for proper *in vitro* development and enhance the success with a nonhuman primate *in vitro* fertilization system. Ethical considerations preclude similar investigations with human embryos. However limited studies of RNA synthesis of unfertilized human oocytes (Hutz *et al.*, 1983) indicate similarity between the human and squirrel monkey oocyte.

The stages of temporal development of *in vitro* fertilized squirrel monkey embryos (table 5) readily indicate the similarity between the squirrel monkey, other nonhuman



primates and the human. These studies amply demonstrate the usefulness of the squirrel monkey model.

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## Luteal function during the periimplantation period and requirement for estrogen for implantation and pregnancy maintenance in the non-human primate

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**Abstract.** An attempt has been made in this paper to review our present understanding of luteal function during the periimplantation period and in particular hormonal requirement for implantation and maintenance of early pregnancy in the non-human primate.

In a fertile cycle the *corpus luteum* is apparently rescued from luteolysis by chorionic gonadotropin secreted by the implanted blastocyst. In the bonnet monkey the serum progesterone titers during the luteal phase of a fertile cycle seems higher compared to that of nonmated cycling monkeys. This suggested that the *corpus luteum* is receiving some stimulatory signal from the blastocyst even prior to implantation. The recent demonstration that human blastocyst in culture secretes into the medium human chorionic gonadotropin essentially support the above assumption. However, attempts to extend the luteal phase of cycling unmated monkeys with exogenous human chorionic gonadotropin injection has hitherto not met with complete success suggesting that there could be other than chorionic gonadotropin, additional luteal stimulatory factors the unimplanted blastocyst is secreting.

*Corpus luteum* is the principle source of both progesterone and estrogen produced during the periimplantation period and dysruption of luteal function, brought about by either lutectomy or ovariectomy or luteinizing hormone antiserum treatment, followed by progesterone supplementation leads to maintenance of pregnancy. This has lead to questioning the need for estrogen in the maintenance of early pregnancy. Recent work using Zuclophiphen, an antiestrogen during days 5-11 of cycle in rhesus monkeys mated between day 9-14, has however, suggested that estrogen may be required for implantation. Further work is needed to arrive at an unequivocal decision regarding the need for estrogen in maintenance of early pregnancy in the primate.

**Keywords.** Estrogen; implantation; *corpus luteum*; non-human primate.

It is presently well accepted that the *corpus luteum* (CL) is essential in the primate for establishment of pregnancy. The CL need apparently ceases once implantation occurs and the placenta takes over the function of producing steroids (Booher *et al.*, 1981). The period between fertilization and implantation in the non-human primate is around 9 days (Hendrickx and Enders, 1980) and limited information is known about the events occurring during the intervening period. The CL of the fertile mated monkey secretes both progesterone and estrogen. Particularly, that the latter is of CL in origin is shown by luteotomizing monkeys. Such monkeys in addition to showing drastic and immediate reduction in both progesterone and estrogen levels do not respond to exogenous luteinizing hormone (LH)/chorionic gonadotropin (CG) treatment (Walsh *et al.*, 1979; Wilks and Noble, 1983; Sheela Rani, C. S., Ravindranath, N., Kotagi, S. G.

Abbreviations used: CL, *Corpus luteum*; LH, luteinizing hormone; hCG, human chorionic gonadotropin.

and Moudgal, N. R., unpublished observations). A comparison of serum progesterone and estrogen levels of cycling fertile and non-fertile bonnet monkeys between days 19–23 of cycle shows that the level of both these steroid hormones is significantly high in the former group (Rao and Moudgal, 1984). Similar observations have been made for other primates, notably the rhesus (Atkinson *et al.* 1975; Hendrickx and Enders, 1980). The increase in progesterone levels of cycling fertile bonnet monkeys even prior to implantation according to Mukku and Moudgal (1979) is perhaps due to the CL receiving some type of signal from the as yet unimplanted blastocyst. The first report that the unimplanted blastocyst could be secreting chorionic gonadotropin was made by Saxena *et al.* (1974) and recently Fishel *et al.* (1984) have obtained confirmatory evidence for this by measuring human chorionic gonadotropin (hCG) in culture medium of human blastocysts. Attempts to extend the luteal phase of cycling unmated monkeys by giving exogenous hCG have, however, generally not met with complete success. hCG given daily either in increasing doses (to simulate pregnancy) or sustaining small doses from day 18–20 of cycle has led to increase in progesterone titers only for a 3–4 day period, the levels falling rapidly thereafter. Continued hCG injection, however, seems to maintain estrogen at high levels, the cycle length itself being extended by a maximum of 5–7 days. These results using both the rhesus (Wilks and Noble, 1983) and the bonnet (Sheela Rani, C. S., Ravindranath, N., Kotagi, S. G. and Moudgal, N. R., unpublished observations) suggest that in addition to hCG, perhaps the CL during the preimplantation period is receiving some other stimulus from the blastocyst to keep producing high titers of progesterone. The nature of this stimulus is yet to be ascertained.

Based essentially on morphological/histological criteria it has been concluded that implantation in most of the non-human primates examined thus far occurs approximately 9 days after fertilization (Hearn, 1980). This day could coincide with the day of CL rescue as determined in non-fertile cycling monkeys (Atkinson *et al.*, 1975). In the bonnet monkeys, based on the *in vitro* sensitivity of CL, removed on days 19, 23, 25 and 28 of cycle to exogenous LH it was concluded that the CL rescue must be occurring on or about day 23, 9–10 days after fertilization (Mukku and Moudgal, 1979). At present other than measurement of elevation in hormone levels (progesterone and CG for example) there is no other way, except by morphological examination, to fix the time of implantation. With the advent of newer protein separation techniques which will permit isolation of specific protein in small quantities (see paper by Roberts in this issue) coupled to radioimmunoassay we can hope that a specific marker protein will be isolated from blastocysts in culture which will permit us to arrive at the time of implantation little more accurately.

Paralleling the increase in CG levels, first detectable in the serum of bonnet monkeys by day 28 of cycle there is a significant increase in both estradiol and testosterone levels (Rao and Moudgal, 1984). While the need for progesterone in implantation and maintenance of pregnancy is fairly well established that of estrogen in these events is questioned. Meyer *et al.* (1969) thus observed using rhesus monkeys ovariectomized on day 6/7 post fertilization that it is possible to obtain implantation and pregnancy maintenance by supplementing with progesterone alone. Bosu and Johansson (1975), however, report that induction of implantation and/or maintenance of pregnancy was poor in ovariectomized monkeys maintained on progesterone alone. Dosing of mated

cycling bonnet monkeys with antisera to oLH or its  $\beta$  subunit (*a/s*) from days 18–20 post fertilization has been shown to reduce drastically serum progesterone and estrogen levels, this leading ultimately to the termination of pregnancy (Prahalada *et al.*, 1975; Moudgal *et al.*, 1978). However, supplementing *a/s* treated monkeys with varying doses of progesterone permits continuation of normal pregnancy suggesting that in the bonnet monkey progesterone alone is adequate to maintain pregnancy during the periimplantation period (Sheela Rani, C. S., Ravindranath, N., Kotagi, S. G. and Moudgal, N. R., unpublished observations).

In the rodents, particularly in rats and mice the requirement for estrogen in the implantation process is well established. Implantation in the hamster, though believed till recently to be solely progesterone dependent has been shown to improve (by increase in the number of implantation sites) with estrogen supplementation (Sengupta *et al.*, 1981). Recent studies have shown that the unimplanted blastocyst (Sengupta *et al.*, 1984) as well as the uterine endometrium have the ability to synthesize estrogen. In the light of this, perhaps some of the experiments done in ovariectomized or *a/s* treated monkeys supplemented with progesterone need to be re-examined. The use of medroxyprogesterone acetate, a non metabolizable progestational compound instead of progesterone in the above studies might provide more definitive result. Alternately a higher titer estrogen antibody or a specific aromatase inhibitor or a chemical analogue having potent antiestrogenic activity will have to be used to establish beyond any doubt the need for estrogen.

Recent work of Adiga *et al.* (1983) provides supporting evidence for the need for estrogen to support pregnancy. They have established that estrogen induces the synthesis of a series of vitamin carrier proteins in both the pregnant rodent and primate (Adiga, P. R., personal communication). Using a pregnant rat as the experimental model they have shown that there is a critical need for these estrogen induced proteins in transporting vitamins across the placenta to the growing foetus, neutralization of these proteins with specific antibodies resulting in the termination of pregnancy. It is, however, not yet clear from their studies, if this 'critical' requirement extends to the primate and if so whether this need is felt during the preimplantation period itself.

The use of a variety of antiestrogens to study the role of estrogens in implantation *per se* in the primate has generally not been successful (Prasad and Sankaran, 1975). This failure has been attributed by some workers to the doses used and in particular to the time of administration of the drug. Assuming that the estrogen surge that occurs at mid cycle may be adequate to sensitize the uterus for subsequent progesterone action, Zuclomiphene, a potent antiestrogen has been administered to rhesus monkeys between days 5–11 of cycle in doses (2 mg/kg/day) that presumably did not effect estrogen function at the hypothalmo-pituitary end. Such a treatment has been shown to render these monkeys infertile (Sankaran M. S., Prahalada S. and Hendrickx A. G., personal communication). Though it is suggested that clomiphene could be acting here as an anti-implantation agent by interfering with the sensitization of uterus to progesterone, the possibility that it could also be acting by bringing about premature expulsion of fertilized ova from the fallopian tube cannot be excluded.

In summary, though the need for a functional CL to support pregnancy during the periimplantation period is well established there is as yet no clear-cut evidence to suggest that estrogen is needed for either implantation or immediate post-implantation

survival of the blastocyst. The work of Sankaran and his co-workers (Sankaran, M. S., Prahalada, S. and Hendrickx, A. G., personal communication) is a pointer in this direction, but further work needs to be done to establish estrogen requirement beyond any doubt. A more accurate biochemical marker for implantation as well as mapping of steroid receptor levels during the periimplantation period in the primate is also required and may together help in better understanding the role of steroid hormones in implantation.

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## Studies on regulation of chorionic gonadotropin secretion in primates

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**Abstract.** The regulation of secretion of chorionic gonadotropin in primates has been studied using both *in vivo* and *in vitro* models. *In vivo* studies using the pregnant bonnet monkey revealed that at the doses tested, the administration of progesterone or estradiol 17 $\beta$  in combination or alone did not result in any appreciable change in the duration or magnitude of serum chorionic gonadotropin levels. However, administration of lutropin-releasing hormone by intravenous route resulted in significant increase in chorionic gonadotropin levels within 30–60 min and the extent of stimulation seemed to depend on the state of pregnancy. For *in vitro* studies, explants or cells prepared from first trimester human placenta has been used. The functional integrity of these cells has been established by demonstrating the binding of [ $^{125}$ I]-labelled human chorionic gonadotropin antibody to the cells as well as the synthesis of [ $^3$ H]-labelled human chorionic gonadotropin. *In vitro* studies using the cells revealed that addition of lutropin-releasing hormone caused a significant increase in chorionic gonadotropin and estradiol 17 $\beta$  secreted into the medium. Thus both *in vivo* and *in vitro* results suggest that lutropin-releasing hormone could be one of the factors involved in regulation of chorionic gonadotropin secretion in primates.

**Keywords.** Chorionic gonadotropin; placenta; lutropin-releasing hormone; regulation; primates.

### Introduction

The placenta in addition to serving the function of transport of metabolites between the maternal and foetal system, also serves as an endocrine gland, by elaborating both protein, peptide and steroid hormones. Of the protein hormones elaborated by primate placenta, chorionic gonadotropin (CG) has been extensively studied, particularly in the case of the human. Available evidence indicates that the primary functions of CG is to extend the life span of the corpus luteum of fertile cycle and maintain its steroidogenic activity. In all the primates studied thus far, the appearance of CG is generally associated with implantation (Hendrickx and Enders, 1980). Serum concentration of CG reach maximal values during early pregnancy and it declines rapidly thereafter, either becoming undetectable as in macaques or maintained at low levels as in human females and a few other non-human primates (Tullner, 1974). Although considerable information has accrued over the years on the chemistry and physiology of CG, very

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Abbreviations used: CG, Chorionic gonadotropin; hCG, human chorionic gonadotropin; LH, luteinizing hormone or lutropin; LHRH, lutropin-releasing hormone; mCG, monkey CG; NBMCS, new born male calf serum; MTP, medical termination of pregnancy; KRBG, Kreb's bicarbonate glucose.



little is known about the factors or mechanism involved in the initiation of CG production, as well as the means by which its continuous production is either shut off or substantially reduced. The present report essentially provides a brief summary of the studies initiated recently to examine the regulation of CG using both *in vivo* and *in vitro* approaches.

## Materials and methods

### Monkeys

For *in vivo* studies, the south Indian bonnet monkey has been used. The details of husbandry, maintenance and breeding has been described in earlier communications (Prahlada *et al.*, 1975; Rao *et al.*, 1984).

### Chemicals

All unlabelled steroids were obtained from Steroloids, New Hampshire, USA. Tritiated leucine (specific activity, 130–190 Ci/mmol), thymidine (specific activity, 70–90 Ci/mmol), progesterone (specific activity, 100–130 Ci/mmol), estradiol 17 $\beta$  (specific activity, 140–170 Ci/mmol) and [ $^{125}$ I]-were obtained from Amersham International, UK. Unlabelled thymidine, leucine and DNA were obtained from Sigma Chemicals Co., St. Louis, Missouri, USA, collagenase from Worthington and Co., Freehold, New Jersey, USA, human chorionic gonadotropin (hCG) (CR 123) and lutropin-releasing hormone (LHRH) were gifts from NIH, Bethesda, Maryland, USA. Ovine luteinizing hormone (LH) and LH $\beta$  were kindly provided by Dr. M. R. Sairam, Clinical Research Institute of Montreal, Montreal, Canada and Dr. C. H. Li, Hormone Research Laboratory, University of California, San Francisco, USA. Tissue culture media were obtained from Himedia, Bombay and disposable culture were from Falcon Plastics, USA. New born male calf serum (NBMCS) was obtained locally and a pooled large batch was sterilised and used in all studies.

### Analytical techniques

Analysis of monkey CG (mCG), progesterone, estradiol 17 $\beta$ , were done according to the procedures validated in the laboratory (Rao *et al.*, 1984). Iodination of hCG was done according to the Chloramine T method of Greenwood *et al.* (1963). For quantitation of hCG in human placental culture samples, radioimmunoassay using rabbit anti-sera to hCG (1:40,000) and [ $^{125}$ I]-hCG was employed. The assay sensitivity was 0.1 ng/ml and intra and inter-assay variations were 6.2% and 10.1% respectively.

### Experimental procedures with monkeys

All injections were given at 10.00 h unless otherwise specified. Blood samples were collected from saphenous vein without using anaesthesia, serum was separated within 6 to 8 h and stored at  $-20^{\circ}\text{C}$  until further processing.

### Isolation of cells from human placenta

First trimester human placenta (before 10 weeks of pregnancy) was collected from cases of medical termination of pregnancy (MTP) from local hospital. The tissue suspended in Kreb's Ringer bicarbonate glucose (1 mg/ml) (KRBG), pH = 7.2, was quickly transported on ice to the laboratory, washed extensively with cold KRBG to remove blood clots and villi were separated out by visual examination. The villi were minced with fine scissors and digested with collagenase (0.1 mg/ml) in KRBG for 10–15 min at 37°C with gentle shaking. After digestion, the tissue suspension was diluted with KRBG and dispersed by drawing it in and out of a sterile plastic syringe connected with a tygon tubing and allowed to settle by standing. Supernatant was carefully removed and filtered through a 100  $\mu$  nylon mesh and the process repeated several times till no tissue remained. Pooled filtrate was centrifuged at 500 *g* at room temperature, pellet was washed once with KRBG and resuspended in minimal volume of Ham F-10 medium containing antibiotics (Penicillin 100 units/ml, Streptopenicillin 100  $\mu$ g/ml, Gentosporin 50  $\mu$ g/ml) and 20% NBMCS. For short term *in vitro* studies, cells ( $1 \times 10^6$  cells/tube in 0.5 ml) were incubated with 1  $\mu$ Ci tritiated leucine or thymidine for 4 h at 37°C in KRBG under 95% oxygen and 5% carbon dioxide and processed for incorporation of label into protein and DNA according to procedures standardised in this laboratory (Sheela Rani and Moudgal, 1977). hCG in the incubation medium was precipitated using a highly potent antisera to hCG in goats.

### Cell culture

For culture,  $5 \times 10^6$  cells in 5 ml of Ham F-10 medium were plated in Falcon petri dishes and maintained under sterile conditions in Forma Water Jacketted Incubator with 5% carbon dioxide at 37°C. Medium (containing 10% NBMCS and antibiotics) was changed every 24 h and hCG, progesterone and estradiol 17 $\beta$  in the medium were analysed by radioimmunoassay. For demonstration of [ $^{125}$ I]-labelled hCG antibody binding to cultured cells, cells were fixed with methanol, acetic acid (3:1) and incubated with [ $^{125}$ I]-labelled antibody in the presence or absence of excess unlabelled goat gamma globulin. [ $^{125}$ I]-was monitored in a PRIA's Packard Counter and  $\beta$ -counting was done in an LKB counter. Statistical analysis was done using Student's 't' test.

## Results

### *In vivo* studies in the pregnant bonnet monkey

**Effect of administration of gonadal steroids on the pattern of CG secretion during early pregnancy in *Macaca radiata*:** It is well known that pituitary gonadotropin secretion is controlled *via* feed back regulation by gonadal steroids. As the placenta produces both gonadotropins and steroid hormones (CG somatomammotropin, progesterone, estradiol 17 $\beta$  and others), it was considered of interest to examine whether the administration of gonadal steroids has any effect on serum CG levels. The experimental design consisted of confirming pregnancy by monitoring CG levels on day 28 of fertile cycle, and injecting progesterone and estradiol 17 $\beta$  thereafter according to protocol, described below. Our earlier studies (Rao *et al.*, 1984) have shown that CG is detectable

by about day 28 of fertile cycle, and that it rapidly reaches peak values by day 35–37 declining thereafter to undetectable levels beyond days 50–55 of fertile cycle. Since the mid-cycle estrogen and LH surges occur in these monkeys on days 8–9 and 10–11 of cycle respectively (Rama Sharma *et al.*, 1978) and the females are mated with breeder males between days 9–14 of cycle. We have considered here day 12 of fertile cycle as day 1 of pregnancy. After confirming pregnancy, the monkeys were injected at 10:00 h i.m. 1 mg of progesterone or 10  $\mu$ g of estradiol 17 $\beta$  or a combination of both in 0.5 ml of groundnut oil per monkey per day from day 30 to day 40 of fertile cycle. Blood samples were collected from day 30 to 60 of fertile cycle and serum CG was determined by radioimmunoassay. The results obtained in the experiment where a combination of progesterone (1 mg) and estradiol 17 $\beta$  (10  $\mu$ g) was administered is presented in figure 1.

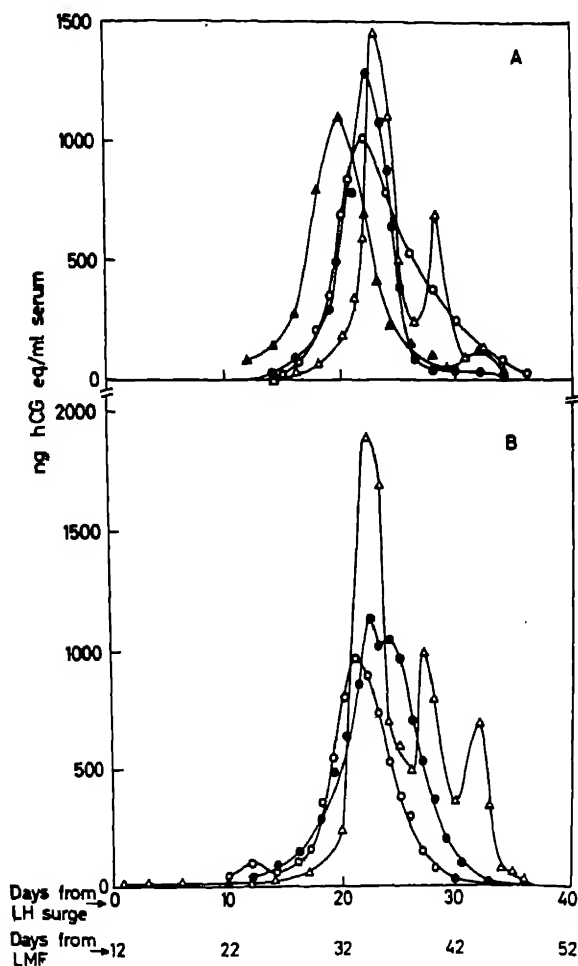


Figure 1. Effect of administration of progesterone (1 mg) and estradiol 17 $\beta$  (10  $\mu$ g) every day from day 30 to 40 of fertile cycle on serum CG levels in pregnant bonnet monkey. A. Depicts the normal profile of CG in four pregnant monkeys. B. Depicts the profile following administration of progesterone and estradiol 17 $\beta$  in another set of three monkeys.

No significant change in the pattern or magnitude of CG levels (figure 1B) during the period under study could be seen when compared with the normal profiles (figure 1A). Essentially similar results were obtained even where each of the steroid hormones were injected separately. The present dose was selected taking into consideration the average animal weight, blood volume and maximal concentration of progesterone seen during early pregnancy in the bonnet monkey. It appears necessary that more studies have to be carried out by injecting different quantities of progesterone and estradiol 17 $\beta$  before a definitive conclusion can be reached.

**Effect of LHRH administration on serum levels of CG during early pregnancy:** The role of LHRH in modulating pituitary gonadotropin secretion is well documented, using a variety of experimental animals. Recent studies have provided evidence that LHRH like material is present in the human placenta (Siler-Khodr and Khodr, 1978). It has also been shown that it is biosynthesised in the placenta (Khodr and Siler-Khodr, 1980) and that addition of LHRH to cultures of human placenta results in an increase in CG released into the medium (Siler-Khodr and Khodr, 1981). In view of this it was considered worthwhile examining the effects of LHRH administration in an *in vivo* model. Two different regimens of LHRH were used. The first one consisted of administering a small dose (10  $\mu$ g) of LHRH by i.m. route in 0.2% gelatin every day from day 30 to day 36 of fertile cycle and analysing daily serum samples for CG. The other approach consisted of giving a bolus of 100  $\mu$ g of LHRH by i.v. route on different days of fertile cycle and analysing blood samples collected at close time intervals for serum CG. The results of these studies are presented in table 1 and figure 2. It can be seen that administration of a low dose of LHRH chronically had no effect on serum CG levels (table 1) as judged by the levels on day 37 of fertile cycle (normally CG levels reach maximal values by this day). However, use of a large dose of LHRH resulted in a 2 to 3 fold increase in serum CG levels within 30–45 min (figure 2). Even here, stimulation was restricted to days 21–40 of pregnancy, no stimulation was seen when LHRH was administered during days 45–50 of fertile cycle. In order to rule out the possibility that the observed increase in CG is not due to interference in the mCG assay employed by pituitary LH released in response to LHRH, a control experiment was carried out. In this, non-pregnant cycling females were given 100  $\mu$ g of LHRH on day 28 of cycle (corresponding to day 16 of pregnancy) and blood withdrawn at different times was

**Table 1.** Effect of administration of low doses of LHRH chronically on serum concentration of mCG in bonnet monkeys.

Group	CG concentration ng hCG eq/ml on day 37 of fertile cycle
	Mean $\pm$ S.E.M.
Control (4)	1242 $\pm$ 214
LHRH (3)	1325 $\pm$ 301*

10  $\mu$ g of LHRH was injected by i.m. route every day from day 30 to day 36 of fertile cycle in 0.2% gelatin.

n of each group is indicated by number in parenthesis.

\* Not significantly different from control.

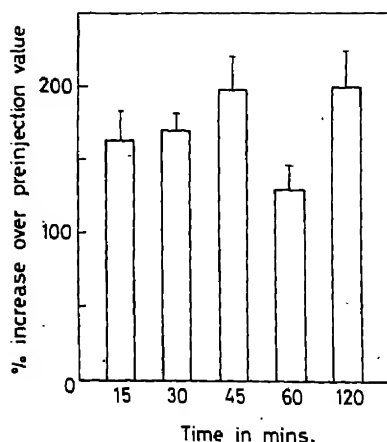


Figure 2. Effect of administration of 100 µg of LHRH by intravenous route to pregnant monkeys.

The stage of pregnancy in animals employed ( $n = 5$ ) ranged from 21 to 40 days. Blood samples were collected prior to injection and at stipulated intervals following injection and serum mCG monitored by radioimmunoassay. Preinjection values were considered as 100% and any change following injection of LHRH expressed as percentage of preinjection value.

analysed for mCG. Inclusion up to 300 µl of serum from these animals did not result in any interference in the mCG radioimmunoassay indicating the specificity of the assay employed. This essentially authenticated the results obtained with LHRH in pregnant monkeys.

#### *In vitro studies using human placental tissue*

As the placental tissue collected at MTP is generally contaminated with blood clots and uterine tissue, one of the first requirements before using the tissue for experiments was to establish the identity and functionality of the cells isolated from the tissue. This was ascertained by demonstrating the presence of hCG as judged by binding of [ $^{125}$ I]-labelled antibody to intracellular hCG as well as precipitation of [ $^3$ H]-leucine labelled/hCG synthesised by the cells. The data provided in table 2 shows that the cultured cells bound significant amount of [ $^{125}$ I]-labelled hCG antibody over the

Table 2. Localisation of hCG in cultured cells isolated from first trimester human placenta.

Group	CPM [ $^{125}$ I]-hCG antibody bound per plate
Non-specific	155510 ± 18335
Specific	347167 ± 29066*

Each value is Mean ± S.E.M. of 4 observations.

\*  $P < 0.005$ .

Cells were cultured for 15 days as described in methods. Medium removed, cells fixed with methanol-acetic acid (3:1) followed by saline wash. In Group I, plates were incubated in the presence of excess of unlabelled goat gamma globulin and [ $^{125}$ I]-labelled gamma globulin prepared from serum of goats immunised with hCG; while Group II was incubated only with [ $^{125}$ I]-labelled anti hCG antibody.

controls and the data presented in table 3 shows the synthesis of [ $^3\text{H}$ ]-leucine labelled hCG by the cells. Also the data presented in table 4 shows the viability of cells as judged by its ability to incorporate [ $^3\text{H}$ ]-leucine into protein and [ $^3\text{H}$ ]-thymidine into DNA.

**Table 3.** Demonstration of hCG synthesis by cells isolated from first trimester human placenta using short term incubation conditions.

Group	CPM [ $^3\text{H}$ ]-leucine incorporation per mg protein
Addition of non-immunised goat serum	12026 $\pm$ 1192
Addition of hCG immunised goat serum	25317 $\pm$ 2761*

Incubation was done at 37°C under 95% oxygen and 5% carbon dioxide for 6 h in the presence of [ $^3\text{H}$ ]-leucine. Medium was processed for labelled hCG synthesised by addition of normal goat serum or goat antiserum to hCG.

Each value is a Mean  $\pm$  S.E.M. of 3 observations.

\*  $P < 0.025$ .

**Table 4.** Experiment to demonstrate the viability of placental cells isolated from first trimester human placenta.

Group	[ $^3\text{H}$ ]-Leucine incorporated into protein CPM/mg protein	[ $^3\text{H}$ ]-Thymidine incorporated into DNA CPM/ $\mu\text{g}$ DNA
Control**	6575 $\pm$ 2403	15 $\pm$ 1.78
Experimental	34929 $\pm$ 3097*	4361 $\pm$ 503*

\*\* Cells incubated with excess of unlabelled leucine or thymidine.

\*  $P < 0.001$  compared to corresponding control.

Cells ( $1 \times 10^6$ ) were incubated at 37°C under 95% oxygen and 5% carbon dioxide for 6 h. Medium was processed for protein and DNA.

#### *Pattern of CG, progesterone and estradiol 17 $\beta$ secretion by cells in culture*

The data presented in figure 3 shows the pattern of CG, P and E secretion into the medium over a period of 12 days. In agreement with other studies, Belleville *et al.* (1978), there is a rapid fall in the quantity of hCG, progesterone and estradiol 17 $\beta$  secreted into the medium within first 48–96 h after which a steady state is maintained indicating that the cells are in a viable state during experimental period.

#### *Effect of addition of LHRH on CG, P and E secretion into medium*

In view of the recent demonstration that the placenta also produces LHRH like material (Siler-Khodr and Khodr, 1978), it was of interest to examine the effects of

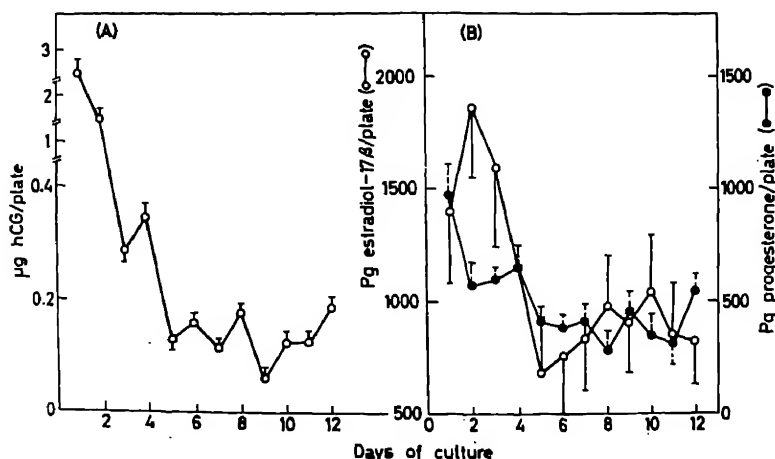


Figure 3. Pattern of secretion of CG (A), progesterone (●) and estradiol 17 $\beta$  (O) (B) by human placental cells in culture.

Cells were isolated and cultured as described in methods. All points are Mean  $\pm$  S.E.M. of 8 observations. Values represent the quantity secreted during a 24 h period.

LHRH on CG secretion under *in vitro* conditions. Addition of LHRH (3.3  $\mu$ g/ml) caused a significant increase in CG and estradiol 17 $\beta$  secreted into the medium (table 5). However, no effect was seen on progesterone concentration in medium. Thus the *in vitro* studies also provide additional evidence for the possible involvement of LHRH in stimulation of CG secretion.

## Discussion

The regulation of pituitary gonadotropin secretion by the modulation of the gonadal steroids *via* LHRH is a well established fact. Functionally the placenta seem to exhibit characteristics of a composite hypothalamus, pituitary and gonad. In view of this it is quite justifiable to expect the placental function to be regulated by a similar mechanism.

Table 5. Effect of addition of LHRH on hCG and estradiol 17 $\beta$  secretion into culture medium by human placental cells.

Group	ng hCG/plate Mean $\pm$ S.E.M.		pg estradiol 17 $\beta$ /plate Mean $\pm$ S.E.M.	
	Control	Experimental	Control	Experimental
Before addition of LHRH	2.13 $\pm$ 0.13	2.15 $\pm$ 0.15	375 $\pm$ 24	383 $\pm$ 16
24 h after addition of LHRH	2.20 $\pm$ 0.52	5.5 $\pm$ 0.2*	241 $\pm$ 86	1150 $\pm$ 279*

Number of plates per group = 3.

\*  $P < 0.05$  compared to value before addition.

LHRH (3.3  $\mu$ g/ml) was added per plate and 24 h later medium analysed for hCG and estradiol 17 $\beta$ , by radioimmunoassay.

This is all the more to be expected for CG as this shares considerable structural and functional homology with LH which is regulated by LHRH. However, our results show that while a negative regulation *i.e.*, feed back inhibition by gonadal steroids does not seem to operate at the doses tried, LHRH seems to have a positive regulatory effect by stimulating CG production. The radioimmunoassay employed for monitoring CG values, has been validated for specificity using sera obtained from non-pregnant monkeys. The serum samples of LHRH injected non-pregnant monkeys have, as expected, been found to have increased serum LH as monitored by the mouse leydig cell assay (unpublished results). It should be pointed out that several studies have reported that CG is not detectable in pregnant rhesus monkeys beyond day 50–65 of fertile cycle (Hodgen, 1980). All the same Siler-Khodr (1979) report that administration of a relatively large dose of LHRH to pregnant rhesus monkeys on day 100 of gestation results in an increase in CG within 15 min. The present studies have been carried out using much lower dose of LHRH and monkeys at a stage of pregnancy when CG is normally secreted.

*In vitro* studies using placental cell cultures have also shown that LHRH stimulates CG, and estradiol 17 $\beta$  secretion into medium. This is in agreement with the studies carried out by Ashitaka *et al.* (1980) and Siler-Khodr and Khodr (1981). In the present studies, analysis of hCG has been carried out by using an antiserum whose specificity towards  $\alpha$  and  $\beta$  subunits was not fully established. Perhaps the use of a characterised antisera to hCG would have provided more valuable information as the available studies on the cell-free synthesis of hCG indicates that the synthesis of  $\beta$  subunit is rate limiting (McQueen *et al.*, 1978). Although our results suggest that LHRH stimulates CG secretion in placenta, it is not clear from these studies whether it stimulates synthesis of both subunits or only one of them or just the release. However, Ashitaka *et al.* (1980) report that while dibutyryl cyclic AMP stimulates only the release, LHRH may stimulate both production and secretion of hCG and its subunits. Studies of Currie *et al.* (1981) have also shown that there are specific receptors for LHRH in human placenta. Das and Talwar (1983) have shown that administration of LHRH agonist during early pregnancy resulted in termination of pregnancy in baboons. Our preliminary studies (unpublished observation) have shown that administration of antiserum to LHRH resulted in a decrease in serum CG levels. All the above cited evidences as well as the results described here clearly suggests that LHRH may be one of the important hormones involved in regulation of CG secretion. It has been suggested that LHRH is produced by the cytotrophoblast and acts on the syncytiotrophoblast which produces both steroids and peptide hormones. Thus while the pregnant monkey model described here provides a convenient way of assessing the activity of LHRH antagonists *in vivo*, the human placental system could perhaps be used for assessing quickly the *in vitro* activity of LHRH antagonists.

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## Post-coital agents and menses inducing drugs

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**Abstract.** The importance of developing of drugs which could be taken post-coitally or used once-a-month in the case of a delay in the onset of the menses is well recognized. The availability of such technology would limit exposure to fertility regulating agents to such occasions where there is coital exposure or possibility of pregnancy.

Methods of post-coital contraception used so far include IUD's inserted post-coitally, estrogens, and combinations of estrogens and gestagens. These are reserved primarily for emergency situations to protect women from unwanted pregnancy resulting from rape or unprotected coitus. Levonorgestrel has shown satisfactory results in terms of contraceptive efficacy and is being further evaluated clinically. A number of problems inherent in the development of post-coital contraception are discussed.

Menstrual regulation could be achieved by a number of approaches: (a) block progesterone receptors and interfere with the preparation of the endometrium for implantation; (b) luteolysis leading to decreased progesterone levels and interruption of implantation; and (c) termination of early pregnancy by prostaglandins. A number of progesterone antagonists have been evaluated. One of the compounds, RU38486 is being evaluated clinically for termination of very early pregnancy.

Deglycosylated derivatives of human chorionic gonadotropin have been shown to antagonize the action of human chorionic gonadotropin and interfere with established pregnancy in rats. Appropriate methods of delivery, immunogenicity and alternate methods for production of human chorionic gonadotropin need to be considered before evaluation of the derivatives for clinical use.

*In vitro* and *in vivo* models need to be developed for evaluation of the teratogenicity and embryotoxicity of post-coital and menses inducing agents.

There are a number of gaps in the knowledge of the processes regulating implantation which should be investigated in rodents and in different non-human primate species.

**Keywords.** Contraceptives; abortifacients; antiprogesterone; antiestrogens.

### Introduction

The importance of the development of drugs which could be taken post-coitally, or used once-a-month in the case of a delay in the onset of menstrual cycles has been stressed by a number of countries (WHO Special Programme in Human Reproduction, Tenth Annual Report, 1981). The relative acceptance of these two approaches to birth control in different populations will, however, be influenced by cultural differences.

### Post-coital agents

There is a felt but as yet unmet need for safe, effective and acceptable post-coital drugs. Methods of post-coital contraception used so far have been reserved primarily for

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Abbreviations used: DES, Diethylstilbesterol; LH, luteinizing hormone; PC, post-coitum; hCG, human chorionic gonadotropin; HF, hydrogenfluoride; DG, deglycosylated.

'emergency situations' to protect women from unwanted pregnancy resulting from rape, a single act of unprotected coitus, failed barrier methods such as ruptured condoms or defective diaphragms, and for the many women who are unprotected because of infrequent coitus.

A 'post-coital pill', by definition, is a drug which is ingested after intercourse takes place (but not necessarily following each coital act on any one day). One of the primary objectives of the post-coital approach is to limit drug exposure, particularly for women who do not have intercourse daily or have a limited number of coital exposures in a month, as compared to the presently available methods of oral contraception in which a tablet containing a combination of a gestagen and estrogen is taken daily for 22 days, long-acting gestagens in which a compound is injected intramuscularly once every two or three months, or IUDs which are inserted by a physician. These methods involve the use of a contraceptive modality unrelated to the act of coitus. Probabilities that an act of intercourse will lead to conception (with establishment of a pregnancy of at least six weeks duration) can be calculated with reference to the estimated day of ovulation. Using self-observed vulvar changes in the characteristics of cervical mucus, a fertile period can be described, in which the last day of 'fertile-type mucus' designated as the 'peak day', has been shown to be correlated closely with the time of ovulation (Billings *et al.*, 1972; Flynn and Lynch, 1976; Hilgers *et al.*, 1978). The fertile period is defined as commencing on the first day of any recognizable mucus, and as ending on the evening of the fourth day after the peak day. In a multicentred study carried out in five centres from four continents, the WHO Task Force on Methods for the Determination of the Fertile Period (1983) has estimated that the probability of conception is low (0.004 per cycle) following the act of intercourse outside the fertile period. It rose to 0.024 on days of 'sticky' mucus four days or more prior to the peak day, to 0.5–0.55 in the three days prior to the peak. It was 0.667 on the peak day, and fell to 0.089 on the third day after the peak. The overall length of the fertile period in the study was  $9.6 \pm 2.6$  (SD) days. It seems reasonable to aim for a post-coital pill that would be taken no more than a maximum of 10–12 days a month. The ultimate objective of a post-coital drug would be to interfere with implantation, irrespective of the day of the cycle when the drug would be taken. A drug effective in inhibiting implantation during the most fertile period is likely to be effective in other periods of the cycle also.

The desirable biological profile of a post-coital drug is difficult to define, since the physiological events that should be interfered with are varied in different stages of the menstrual cycle. No single drug is likely to affect all the physiological mechanisms during the different phases of the menstrual cycle. Some oral contraceptives (combinations of gestagen and estrogen) are effective by inhibiting ovulation. A post-coital drug is intended to act, among other ways, by inhibition of the processes crucial to the initiation and establishment of pregnancy *viz.* the implantation of the blastocyst in the uterus. It is clear that the post-coital pill would be needed most in the periovulatory period when the chances of conception are highest.

The problems concerning the use of the post-coital pill are the: (a) number of times the pill is taken during a cycle; (b) side effects depending on the number of days of coital exposure and tablets taken; (c) variation of side effects according to the stage of the cycle (follicular, ovulatory, or luteal phase) when the pills are ingested, *e.g.* a compound with gestagenic properties when taken a number of times during the luteal phase of the

menstrual cycle may delay the onset of menstruation and result in heavy bleeding, whereas if taken a few times during the follicular phase of the cycle it may lead to spotting or progesterone withdrawal bleeding.

#### *Clinical studies*

Two approaches to post-coital contraception, used as "emergency" measures involve: (a) insertion of a copper containing intrauterine device, the copper T, within five days of unprotected coital exposure; no pregnancies were reported in a series on 97 women treated in this way (Lippes *et al.*, 1976); or (b) administration of synthetic, natural or conjugated estrogens. Diethylstilbesterol (DES), has been used at a dose of 50 mg daily in divided doses for five days with a failure rate from 0 to 2.4%. The effectiveness of this method depends partly on its early administration, within 24–72 h after coitus (Kuchera, 1975). Ethinyl estradiol and other conjugated estrogens have also been evaluated with varying success. The action of these steroids appears to be due to changes in the endometrium rendering it hostile to implantation (Yuzpe, 1979). Synthetic estrogens like DES are no longer used and have been associated with adverse effects on the reproductive tract in male and female progeny (Stilman, 1982). One of the major handicaps in the development of post-coital drugs has been the difficulty of completely dissociating the oestrogenicity of available compounds from their anti-implantation effect. A number of non-steroidal compounds have been evaluated in animals in an effort to identify an effective drug having little estrogenic activity, but none has reached the stage of clinical trials (Harper, 1982; Prasad and Sankaran, 1975).

A combination of 200 µg of ethinyl estradiol and 2 mg of dl-norgestrel seems to be effective as an emergency post-coital contraceptive with several advantages over estrogens alone. The combination was administered either as a single dose (Van Santen and Haspels, 1982) or in two doses (12 h apart) after an unprotected coitus within the previous 72 h (Yuzpe *et al.*, 1982). In a multicentred study involving 692 women the pregnancy rate was 1.6%; no serious side effects other than nausea/vomiting were encountered and menstrual bleeding occurred in more than 98% of treated subjects within 21 days after treatment. Because of the potential failure rate and unpredictability of ensuring menstrual bleeding, Yuzpe *et al.* (1982) suggest that the method be reserved as an emergency post-coital contraceptive only.

Randomized double-blind studies are necessary to further compare the efficacy of this form of therapy with ethinyl estradiol or conjugated estrogen employed alone. It has been speculated that the combination acts either by: (a) suppressing ovulation; (b) disrupting luteal function by acting directly on the corpus luteum; or (c) interfering with appropriate endometrial responses to ovarian steroids.

#### *Progestins*

The two most frequently used compounds include d-norgestrel and quingestanol acetate (Mischler *et al.*, 1974); the latter is no longer in use.

Post-coital administration of levonorgestrel has shown satisfactory results in terms of contraceptive efficacy, tolerance and acceptability. A dose-response relationship has been reported in a number of studies (Kessuru *et al.*, 1974; Kovacs *et al.*, 1979); the use of 150 µg dose was associated with a high failure rate, whereas 750 µg of levonorgestrel administered within 3 h after coital exposure was highly effective as a contraceptive

(Farkas, 1978); four pregnancies occurred in a two and a half year study involving 111 women treated during 999 cycles. In another study, when the drug was administered after coitus only during the periovulatory period in 50 women (150 cycles), there was no pregnancy (Kovacs *et al.*, 1979). Although the study does not reflect on the real-life situation, i.e. restricting coitus only to the periovulatory period, it does indicate the efficacy of the drug when administered during the most fertile period. Levonorgestrel (Postinor) is marketed in Hungary as a post-coital drug, subject to a maximum intake of four tablets (each of 750  $\mu\text{g}$ ) a month, i.e. 3 mg/total dose/month. Depending on the dose employed, nausea and vomiting, headache, dizziness and breast tenderness have been reported. However, one of the most frequent problems is the alteration in the menstrual pattern produced by the progestin. Irregular menstrual bleeding does not appear to be acceptable to many cultures, and if amenorrhoea results, it is necessary to rule out the possibility of pregnancy before continued use of the progestin. The WHO is carrying out a multicentre study in eight countries to evaluate the post-coital efficacy of 750  $\mu\text{g}$  of levonorgestrel when administered during the periovulatory or fertile period only. The other objectives of the study are (a) to assess the side-effects of the drug and to correlate these, if possible, with the dose of the drug administered, as well as the timing of its administration in relation to the menstrual cycle, and (b) determine the acceptability of such a regime (WHO Special Programme of Research in Human Reproduction, Eleventh Annual Report, 1982).

### *Home visiting pill*

Nine home visiting antifertility pills or vacation pills have been developed and used as post-coital or anti-implantation agents in the People's Republic of China (Lei and Hu, 1981); of these, anordrin (at a dose of 7.5 mg) apparently used as post-coital pill interferes with implantation by inhibition of luteal function and of endometrial development. It is not clear from the Chinese studies if anordrin was even used strictly as a post-coital pill. The antifertility activity of anordrin has been attributed to its antiestrogenic activity (Mehta *et al.*, 1981), or to estrogenic acceleration of tubal transport or degeneration of eggs during tubal transport in the hamster (Gu and Chang, 1979).

A number of attempts have been made to synthesize related compounds to dissociate the estrogenic activity from antifertility activity but none had yet reached the stage of clinical evaluation.

### *Centchroman*

The biological and antifertility effects of centchroman (3,4-trans-2, 2-dimethyl-3-phenyl-4-*p*-( $\beta$ -pyrrolidinoethoxy)-phenyl 7-methoxychroman have been investigated (Kamboj *et al.*, 1977); the compound has weak estrogenic and potent anti-estrogenic activity; it is devoid of progestational androgenic and anti-androgenic properties but antagonises progesterone in the rabbit. Its antifertility activity in rats, dogs and monkeys may be due to its multiple hormone attributes such as estrogenic, anti-estrogenic and antiprogestational activities.

Phase II clinical studies have been carried out in post-coital (60 mg) and once-a-week treatment schedules at doses of 120, 60, 30, 25, 20 and 15 mg. The results reported

licate that centchroman provides good protection against pregnancy at 30 mg and her higher doses studied (Nitya Anand, personal communication). Ovarian and erine enlargement was observed in both treatment regimes, with recovery to normal e within 30 days of withdrawal of the drug. Delay in menstruation of varying ration has also been reported. Since the doses used led to ovarian/uterine largement and irregular cycles, smaller doses (30 mg/week) are being evaluated nically. As centchroman has been shown to induce release of luteinizing hormone (H) in rats (Arabatti *et al.*, 1977) the possibility of induction of ovulation as with omiphenes cannot be ruled out. The reported side effects on the ovary, uterus, and the e regular menstrual cycle caused by centchroman, call for further studies with much wer doses than used so far, before the hopes raised of the compound being used as a ost-coital or once-a-week drug can be realized.

## valuation of post-coital drugs

### animal models

primary problem is the animal model for evaluation of post-coital drugs. By e definition, such drugs should be evaluated for their efficacy by administration after ating. However, a post-coital drug can be tested in a rodent or non-human primate odel only during the periovulatory period, when the females are sexually receptive nd mate. Since mating does not occur at other times of the cycle, as is the case in the uman, there is no possibility of testing the post-coital efficacy of drugs in any animal odel by administration during periods of the menstrual/estrus cycle other than the eriovulatory period. A possible, but tedious approach is to artificially inseminate boratory rodents/non-human primates at other periods of the cycle and administer e drug to determine the side effects.

### toxicology

There are no standard procedures set out for the toxicological evaluation of post-coital rugs in animals. The questions that need to be answered are (a) how many times in a onth should the drug be administered, and (b) what should be the frequency of dministration? For example, for levonorgestrel which is administered as a post-coital gent (Postinor) in Hungary, the maximum suggested intake is 4 tablets/month, *i.e.* 50 µg/tablet, equivalent to a total dose of 3 mg/month. The question is, should the oxicology of such a drug be carried out by administering the test compound to animals rodents or non-human primates) using the same schedule as would be applicable to the uman, or should the drug be administered daily and, if so, for how long? This is true or evaluation of menses-inducing drugs also.

## Problems associated with post-coital contraceptives

Yuzpe (1979) has summarized very well the problems associated with post-coital ontraceptive studies, as follows.

Ideally, post-coital evaluation studies should be carried out in randomized, double-

blind fashion, with couples of proven fertility who will utilize the specific technique (or a placebo) once at mid-cycle only when the risk of pregnancy is at a maximum, and who will avoid coitus at all other times during that cycle. However, it is obvious that the ethical considerations of such studies make it difficult to obtain volunteers for this purpose. For this reason, studies are generally carried out upon women who are exposed at various times during the menstrual cycle. Thus, the number of women evaluated for a particular method must be sufficiently large to provide statistically significant data regarding efficacy.

Studies which utilize patient volunteers whose admission to only one single act of unprotected coitus is necessary for study inclusion are at a disadvantage. Reliance upon the patient to take the entire dosage of prescribed medication also has its obvious disadvantages, especially if nausea and vomiting are common occurrences.

#### *Risks of post-coital contraception*

In the light of potential failure of this technique, back-up abortion facilities should be available. The true potential of the adverse effects of treatment failures is, as yet, not fully understood. Furthermore, failure in such instances should be considered as a method failure similar to pregnancy resulting from the failure of an IUD or other barrier method.

#### *Benefits of post-coital contraception*

The major value of post-coital contraception in countries like North America seems to be in the area of emergency treatment. The woman at risk of pregnancy from sexual assault or a single, unprotected coital exposure may be spared the emotional and physical trauma of unwanted pregnancy.

Once the purpose has been achieved, the patient may be counselled, and her choice of contraception made from one of the available methods. This choice, of course, depends upon patient motivation, coital frequency, socioeconomic status, as well as other sociologic variables.

Easily available post-coital contraception also has the potential of reducing the use and acceptability of cyclic oral contraception, IUDs and barrier methods which have proved so effective for millions of women.

If a simple effective method of post-coital contraception is available, the woman may be spared the psychic trauma which is often associated with awaiting the passage of time in anticipation of the next expected menses.

#### **Menses-inducing drugs**

There is great demand from women and from national family planning programmes for a simple and safe method of birth control for use when menses are delayed for a few days. The availability of such technology would limit exposure to fertility regulating methods only to such occasions when there is a probability of pregnancy. Moreover, if fertilization had indeed, occurred, interruption would take place at the earliest stage of pregnancy, thereby reducing the excessive bleeding encountered with later termination of pregnancy.

The only currently available methods of termination of very early pregnancy are mechanical aspirators that in most instances require trained personnel. Experience with them indicates an efficacy of 95% when used at five or six weeks since the last menstrual period. About 5% of patients have severe bleeding or retention of products of pregnancy requiring vacuum aspiration. Uterine perforation rates are approximately equivalent to those at seven to eight weeks vacuum aspiration.

Menstrual regulation could be achieved by any one or more of the following approaches:

- block progesterone receptors and interfere with the preparation of the endometrium for implantation;
- luteolysis leading to decreased progesterone levels and interruption of pregnancy;
- termination of early pregnancy by prostaglandins.

A study comparing the efficacy and safety of two prostaglandin E<sub>2</sub> analogues and vacuum aspiration for termination of very early pregnancy in women with a delay of menstruation of up to 14 days is ongoing at present in several countries (WHO Special Programme of Research in Human Reproduction, Eleventh Annual Report, 1982; 1983).

#### *Progesterone receptor blockers—progesterone antagonists*

Progesterone plays an indispensable role during the implantation phase and early pregnancy in animals and women; withdrawal of progesterone leads to breakdown of the secretory endometrium in the normal menstrual cycle. Likewise, the decidua which develops from the endometrium following implantation regresses and is shed in the absence of progesterone. There are several possible methods for interference with the secretion and action of progesterone. One of these is to block the action of progesterone on the endometrium.

It has been demonstrated that progesterone receptors are generated during the follicular phase of the menstrual cycle reaching a maximum just before ovulation, after which they decline markedly (Bayard *et al.*, 1975). During the luteal phase of the menstrual cycle, despite the decrease in the total concentration of progesterone receptors in the endometrial cells, there is an increase in their concentration in the cellular nuclei (Bayard *et al.*, 1979; Levy *et al.*, 1980). Early pregnancy endometrium (8–10 weeks gestation) is characterized by a large concentration of progesterone receptors, exceeding those of any period in the menstrual cycle (Levy *et al.*, 1980). A progesterone antagonist, or antiprogesterin, taken orally during very early pregnancy or after missed menses could, by binding to endometrial progesterone receptors, interfere with the maintenance of the secretory endometrium essential for continuation of pregnancy. A number of compounds evaluated so far have shown either agonistic (progestational) and/or estrogenic activity.

The desired characteristics of a progesterone antagonist are that it should: (a) be orally active; (b) have a high affinity for progesterone receptors in the radio-receptor binding assay in competition with progesterone. There is no agreement on whether the ideal compound would bind rapidly and dissociate slowly or bind and dissociate rapidly; (c) have high antiprogesterin and low progestin activity in the Clauberg test in



rabbits, in decidualoma test in rats, in pregnancy maintenance test in rats and in the diamine oxidase assay; (d) have no or markedly reduced estrogenicity; (e) show significant anti-implantation effect in rats/hamsters/rabbits and non-human primates; and (f) be free from toxicological and teratological effects.

Over the past decade, attention has been directed towards understanding the molecular basis for the biological activity of progesterone in order to permit the development of an anti-progestational agent. Most of these efforts have been empirical. Investigators have examined the steroids that have progestational activity in the hope of defining the molecular requirements for activity and structural features that could be manipulated to alter the degree of response. It is now clear that knowledge of the structures of the progestins alone will not permit the development of an antiprogestin.

The WHO Task Force on Post-coital and Once-a-Month Drugs has embarked on the synthesis of new progesterone receptor blockers based on the study of the structure of the uterine progesterone receptor. The human endometrial progesterone receptor has been purified (Smith *et al.*, 1982). Three active sites of the receptor have been identified by affinity labelling with 21-, 16- and 11-O<sup>3</sup>H bromo-acetoxypregesterone; these three derivatives specifically bound to and displaced progesterone bound to the human endometrial progesterone receptor and migrated as a single protein band on polyacrylamide gel electrophoresis of molecular weight 42,000 (Holmes *et al.*, 1981).

A few questions which need to be answered are:

What does the binding site look like? Exactly what happens to the site when progesterone binds to it? Do compounds with vastly different progestational activity trigger different degrees of change in the receptor? To what extent is the steroid buried in the receptors? Does part of the steroid control binding and another part control activity? Is the steroid partially exposed when bound to the receptor? Does this exposed part control activity by interaction with another macromolecule, histones, non-histones or DNA? Can we design a molecule that will fulfil the structural requirements for binding but fail to elicit the activating response?

The logical way to obtain answers to these questions is to determine the X-ray crystal structure of the progesterone receptor. Once the receptor protein has been crystallized, a series of steroidal and non-steroidal compounds can be examined to determine how they fit into the receptor site.

Thus the scientific rationale for such an approach for development of antiprogestins is clear. However, it does require large amounts of financial investment.

Herrmann *et al.* (1982) have described the use of a 19-nor testosterone derivative RU38486, 17 $\alpha$ -propynyl-11 $\beta$ -(4-dimethyl-aminophenyl)-, 9,10-dihydro-19-nor testosterone for induction of abortion and menses regulation. This compound has great affinity for the progesterone receptor without progestational, estrogenic or anti-estrogenic activity. It binds to glucocorticoid and human progesterone receptors. No toxic effects were seen during 30 days of administration to rats and monkeys except for those attributable to the antiglucocorticoid effects in high doses. When 200 mg/day of the compound was given, either in two or four divided doses to women who were 6-7.5 weeks pregnant, abortion occurred in 8 of the 11 subjects in 3-8 days after the initiation of treatment. Six women with normal menstrual cycles were administered 50 mg/day for four days between days 22-25 of the cycle and in all cases bleeding occurred within 48 h of treatment concurrent with a rapid fall in plasma progesterone and estradiol to

follicular phase levels. No side effects were reported in any of the eight treatment cycles and the subsequent menstrual cycles were apparently ovulatory as judged by basal body temperature. These results are highly encouraging in demonstrating clinical effectiveness of an antiprogestational agent. The WHO Special Programme in Human Reproduction is carrying out a dose finding study of the compound for termination of very early pregnancy (delayed menses) (WHO, 1983). Even as these clinical studies progress, attempts to synthesize even more potent and active progesterone antagonists continue based on a better understanding of the receptor binding sites of the human endometrial progesterone receptor.

The objective in the development of such compounds would be to use them regularly once-a-month for menstrual regulation or for termination of very early pregnancy.

#### *Other compounds*

A possible anti-implantation agent is  $\alpha$ -difluoromethylornithine which inhibits implantation in rats when administered intraperitoneally at a dose of 200 mg/kg twice daily on day 4-7 of pregnancy (Reddy and Rukmini, 1981). It is also effective in rabbits (Fozzard *et al.*, 1980). The mechanism of action of the compound is by inhibition of ornithine decarboxylase enzyme and consequently the synthesis of putrescine, which is essential for embryogenesis (Fozzard *et al.*, 1980). The compound is active *in vivo* and apparently non-toxic (Prakash *et al.*, 1978). In view of the possible consequences of systemic inhibition of a ubiquitous enzyme like ornithine decarboxylase, the potential of its use for fertility regulation remains to be determined.

An anti-progesterone monoclonal antibody, has been shown to block the establishment of pregnancy in mice, probably by interfering with the transport of the fertilized ovum along the fallopian tube when administered at 32 h post-coitum (PC) and interrupted implantation when injected 109-130 h PC (Wright *et al.*, 1982); these effects were possibly due to reduction by the antiprogesterone antibody of readily available progesterone in circulation by more than 85 % during the critical phase of implantation. The acceptability and safety of a passive immunization method needs to be established before its use for inhibition of implantation. Passive immunization even with monospecific antibodies runs the risk of sensitizing the recipient to the foreign antibody protein. Although in theory this risk could be reduced by 'typing' the recipients and the clone products, this is unlikely to prove practical or entirely feasible for wide-scale application. Another way in which the risk of sensitization can be reduced is by the use of  $F(ab)_2$  fragments although this would add an expensive additional step in the preparation. Even if the intended use is as a monthly administration, or at less frequent intervals, the risk of sensitization would still remain.

#### *Zoapatle*

Zoapatle, is the common name for *Montanoa tomentosa*, which grows in Mexico. Ethnobotanical studies and the clinical use of a number of constituents extracted from the plant have been reviewed recently (Gallegos, 1983); extracts from zoapatle have been shown to be uterotonic (Gallegos, 1983); bleeding occurred in four out of six subjects when administered at a dose of 100 g/day during early pregnancy (Landgren *et al.*, 1979). Zoapatanol, however, which is obtained by total synthesis, has apparently no

uterotonic effect (Smith *et al.*, 1981). This discrepancy between the plant extract and the pure synthetic compound needs to be resolved. A number of other chemical entities extracted from zoapatle such as kauradienoic acid, kaurenoic acid, zoapatlin and monoginoic acid need to be evaluated for their biological activity, in addition to continuation of studies with pure zoapatanol (Gallegos, 1983). The question that needs to be considered is whether optimal effects are produced by combination of one or more of the above constituents.

### Interference with luteal function

Normal corpus luteum function and secretion of progesterone are needed for implantation to occur and pregnancy to be established. Its function is directly under the control of pituitary gonadotropins and in a non-pregnant menstrual cycle the corpus luteum regresses within 10–12 days after ovulation. Human chorionic gonadotropin (hCG) secreted by the trophoblast of the developing and implanting blastocyst, rescues the corpus luteum from regression and maintains it.

#### *hCG derivatives*

Attempts have been made to prepare derivatives of hCG which could act as antagonists of hCG for inhibition of luteal function. Such an hCG antagonist when administered after missed menses could competitively displace hCG bound to the corpus luteum leading to luteal regression, decreased progesterone levels, interruption of pregnancy and induction of menstrual-like bleeding (WHO Special Programme of Research in Human Reproduction, Annual Reports, 1979–1983). A number of derivatives of hCG have been prepared and their physico-chemical characteristics and biological activities evaluated *in vitro* and *in vivo*. One of the derivatives, periodate oxidized–reduced derivative, designated PORA-hCG which had lost all its galactosyl and 40% of mannosyl residues, following sequential treatment with specific exoglycosidases has been evaluated (Bahl and Moyle, 1978). This derivative bound to the ovarian receptors with a 2.4 times higher affinity than hCG, caused a dose-dependent inhibition of the action of hCG in production of cAMP by corpus luteal cells *in vitro*, had 2–5% of agonistic activity in stimulating progesterone production (Kalyan *et al.*, 1982; Channing and Bahl, 1978a,b); PORA-hCG inhibited implantation in rats when administered subcutaneously at doses greater than 0.25 µg/day from days 1–5 PC (Kalyan *et al.*, 1981). However, one of the principal disadvantages is that the derivative is stable only at –20°C in the lyophilized or solution form (Kalyan *et al.*, 1982).

A different approach for the production of hCG antagonists has been reported by Manjanath and Sairam (1982) who have shown that treatment of purified hCG with anhydrous hydrogenfluoride (HF) at 0°C for 60 min removed 75% carbohydrate without affecting the peptide moiety. The O-glycosidically-linked glycopeptide part in the C-terminal peptide of the  $\alpha$ -subunit was least affected by treatment. Deglycosylation reduced heterogeneity that is inherent to native hCG. Its solubility was not affected. The product was obtained in good yield in a stable and lyophilized form. Even the most drastic conditions could not remove all of the carbohydrate; this may even lead to reduction of receptor binding and antagonistic activities. In cell receptor

binding assays, viz. rat testis, pseudopregnant rat ovary, porcine granulosa cell, rhesus monkey testis, deglycosylated (DG) hCG showed approximately 200% activity as compared to native hCG. There was no reduction in immunological activity of DG hCG as tested by radioimmunoassay using [ $^{125}$ I]-hCG and an antibody to native hCG. As against full agonistic activity in receptor binding, DG hCG almost completely lost its activity in responsive cells incubated *in vitro*. Both cyclic AMP accumulation and testosterone synthesis caused by native hCG were inhibited by the concomitant presence of DG hCG (Sairam, 1982).

DG hCG is heat stable. Significant receptor binding, immunological and hormonal antagonistic properties are retained by DG hCG after exposure in a boiling water bath. This may contribute to the long shelf life of the antagonist in aqueous solutions; it is stable in the lyophilized form at 4°C for up to 12–16 months (Sairam and Manjanath, 1983). DG hCG has been shown to inhibit implantation when administered subcutaneously at a dose of 50 µg/day from days 1–5 PC; similar doses administered between days 8–11 of pregnancy resulted in complete termination of pregnancy and foetal resorption accompanied by a fall in serum progesterone levels. The derivative had little effect on pregnancy, foetal growth or parturition when administered from days 13–16 (Kato *et al.*, 1983).

A number of issues that need to be studied before these derivatives may be considered for use in termination of early pregnancy are: (a) immunogenicity of DG hCG; (b) antifertility effects in non-human primates; (c) alternate methods of derivative production; and (d) delivery system and mode and route of administration.

#### *Evaluation of menses-inducing drugs*

The efficacy of menses-inducing drugs (anti-progestins or luteolytic agents) can be evaluated in non-human primates in which pregnancy can be determined accurately by timed mating. Reproductive profiles and standard criteria for the choice of females for such studies have been reported for the rhesus monkey, bonnet monkey and the baboon (Anand Kumar *et al.*, 1980; Hendrickx and Enders, 1980; Murthy *et al.*, 1980). The test drugs are administered on days 22–24 of the menstrual cycle in animals in which pregnancy is established by monitoring levels of chorionic gonadotropin and the effects evaluated by termination of pregnancy, onset of menstrual bleeding and decrease in serum levels of chorionic gonadotropin to non-detectable levels.

The luteolytic activity of compounds can also be evaluated in monkeys by administration of the compound during the luteal phase of non-pregnant rhesus monkeys (Ricardo *et al.*, 1982). One of the principal disadvantages in these studies is the short luteal phase. If the functional life of the corpus luteum of a non-pregnant cycle can be prolonged for 10–15 days by administration of hCG, it should provide a suitable model to evaluate the efficacy of luteolytic agents. Such studies are in progress in the bonnet monkey (WHO 1982).

#### *Research on implantation in non-human primates*

**Development of primate models:** There are a number of gaps in the knowledge of the processes regulating implantation which need to be investigated. Some of these are:

- hormonal profiles during the periimplantation period
- changes in the concentration of progesterone and estrogen receptors during the periimplantation and post-implantation periods;
- hormonal requirements for ovum implantation;
- changes in histochemistry of the endometrium during implantation: whether there are any enzymes which are crucial to the initiation of implantation;
- role of the blastocyst in initiating metabolic and cellular changes in the endometrium at the time of implantation;
- whether the functional life of the corpus luteum of a non-pregnant menstrual cycle can be prolonged by hCG administration, if so, for how long? Such a model would be useful for screening luteolytic agents.

These studies could provide new leads for evaluation and development of new anti-implantation agents which could be used either PC or for menses-induction. There is no agreement on the appropriate primate species to be used for these studies. Such studies should be carried out in a range of primate species and not concentrated on the few on which data is currently available, since it is most unlikely that any one species will prove ideal as a model for all aspects of implantation in the human. There are also other factors on the availability, cost, ease of management, breeding performance in captivity. That might affect their choice for achieving different goals in research on implantation.

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## **Trends in blastocyst research with relevance to development of contraceptives: General discussion.**

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The Indo-US symposium on Blastocyst Research concluded with a round table discussion on trends in research with particular relevance to the development of contraceptives. A number of events crucial to the initiation and maintenance of implantation were identified and possibilities of developing a contraceptive modality based on interference with these events in implantation were discussed.

There is a great demand from women and national family planning programmes for simple, effective and safe methods of fertility regulation which can be self-administered either post-coitally or for use when menses are delayed by a few days. The availability of such contraceptive technology would limit exposure to fertility regulating agents only to such occasions when coitus takes place or when there is a probability of pregnancy. Moreover, if fertilization had indeed occurred, interruption would take place at the earliest stage of pregnancy thereby reducing the excessive bleeding encountered with later termination of pregnancy. Methods of post-coital contraception used so far have been reserved primarily for emergency situations to protect women from unwanted pregnancy resulting from rape or acts of unprotected coitus or failed barrier methods.

Probabilities that an act of coitus will lead to conception, with reference to the estimated time of ovulation, is highest during the fertile period ( $9.6 \pm 2.6$  days). The objective of any form of contraception related to coitus would be to interfere with implantation irrespective of the day when the drug is taken. A drug effective in inhibiting implantation during the most fertile period is likely to be effective in other periods of the menstrual cycle also.

The areas selected for discussion were:

### **Uterine sensitivity**

- What are the factors that regulate uterine sensitivity and maternal recognition of the blastocyst?
- What are the factors involved in the development of uterine refractoriness? Can refractoriness be induced?
- Are there specific uterine and blastocyst proteins involved in implantation?
- Is induction of a short luteal phase a possible approach to modify uterine environment and render it hostile for implantation?



## Blastocyst

—What is the role of estrogens (ovarian or produced by the blastocyst) in implantation? Can this be interfered with by antiestrogens administered systemically? What is the role of catechol estrogens in implantation?

## Luteolysis

Research needs, animal models, status of R and D with methods to inhibit implantation and disruption of established implantation.

### *Uterine sensitivity:*

Initiation of implantation is dependent on a coordinated series of events in the uterus triggered by the sequential action of estrogens and progesterone secreted by the ovary during the pre, peri and post-ovulatory period. Uterine sensitivity and receptivity to implantation is variable in different species and is perhaps less than two days during the fertile period in the human. At other times in the menstrual cycle, the uterine environment is hostile to the implantation of the blastocyst. Refractoriness is a condition when the process of decidualization does not occur in rodents. The biochemical basis for the development of uterine sensitivity/refractoriness is not fully understood in non-human primates and human. The generation, storage and release of vasodilatory mediators, catechol amines (endometrial monoamine oxidase and catechol-O-methyl transferase involved in catechol amine deactivation), prostaglandins, cathepsins, phospholipase A etc., appear to be involved in the modulation of uterine vascular functions and uterine sensitivity. The crucial question is the identification of factor/s involved in the onset of uterine refractoriness and whether such a condition can be induced. Recent studies in baboons have led to the isolation of a low molecular weight substance (400–500 daltons) from the refractory uterus which appears to be toxic. Further studies are required to confirm and extend these observations in other non-human primates and human. Biochemical marker/s for the recognition of the refractory period need to be identified.

The uterine epithelium and subepithelial stroma destined to decidualize show a differential temporal response to the action of estrogen and progesterone. A number of proteins synthesized by the luminal epithelial cells in response to estrogens to achieve sensitivity have been identified but the specificity of any of these to implantation is not established. Likewise, a number of proteins secreted by the unimplanted blastocyst have been isolated, but the role of none of these, other than human chorionic gonadotropin (hCG), has been clarified. It is unlikely that any of the presently known uterine/blastocyst proteins can be used to develop an immunological approach to interfere with implantation. hCG subunits or smaller peptides of hCG constitute the basis for the only immunological approach to interfere with implantation which is under development. There is need for the development of an *in-vitro* model to culture the endometrium in different phases of the menstrual cycle with the blastocyst to elucidate the biochemical entities involved in the interaction between the two. Such a model would also permit analysis of the sequential events and concurrent biochemical

changes associated with implantation, *viz*, apposition, adhesion and penetration.

It is desirable to modify uterine sensitivity to implantation by simulating naturally occurring condition of 'short luteal phase' which is generally associated with infertile cycles. Such a condition has been induced in the rhesus monkey by reduction of follicular stimulating hormone (FSH) in the follicular phase. This leads to disruption of the luteal phase and reduction in levels of progesterone. This is an area in which further work is needed.

One of the methods to modify uterine sensitivity is to interfere with the action of progesterone on the endometrium. A progesterone antagonist or antiprogestin, taken orally during very early pregnancy or after missed menses could, by binding to endometrial progesterone receptors, interfere with the maintenance of the secretory endometrium essential for the continuation of pregnancy. One of these compounds, RU-38486 has received considerable attention recently and is being evaluated clinically. An antiprogestone monoclonal antibody has been shown to inhibit implantation in mice possibly due to the reduction by the progesterone antibody of readily available progesterone in circulation for maintaining uterine sensitivity for implantation. The acceptability/safety of a passive immunization method and its efficacy in other species remains to be established.

### *Blastocyst*

Interaction between the blastocyst and uterus is essential for the initiation and maintenance of implantation. The role of estrogen produced by the ovary (which appears in the form of a second peak during the week following ovulation in women) to the initiation of implantation is not clear. Estrogen of embryonic/endometrial origin has been implicated in the process of maternal recognition in several species including the rat, mouse, rabbit, pig and hamster but remains to be demonstrated in the non-human primates and human. Implantation in the hamster is considered to be solely dependent on progesterone but it has recently been shown that the number of implantation sites increase with estrogen supplementation. The steroidogenic activity of the embryo and the endometrium shows wide qualitative and quantitative species variations. The role of these local estrogens at the time of implantation is not clear. The blastocyst secretes several proteins besides estrogens, *viz*, proteins (hCG is the most well defined), catechol estrogens and several enzymes. A specific ovine trophoblastic protein besides prolonging the function of the corpus luteum, interacts with the endometrium by altering the pattern of protein synthesis by endometrial explants cultured *in vitro*. The presence and role of similar proteins in non-human primates remain to be established.

Steroidal and non-steroidal antiestrogens administered systemically post-coitally inhibit implantation in the rat but are ineffective in the hamsters and primates. However, antiestrogens administered intraluminally into the uterine lumen post-coitally interfere with implantation in the hamster, a species which is not dependent on ovarian estrogen for implantation. This has been interpreted as evidence for the role of estrogen secreted by the blastocyst in triggering implantation in the hamster. The failure of antiestrogens to inhibit implantation in non-human primates may be due to the insufficient dose and/or time of administration of the compounds. Zuclomiphene, a

potent antiestrogen, administered to rhesus monkeys between days 5–11 of the cycle at doses which do not affect hypothalamic function, interfere with fertility of the treated females. It is necessary to evaluate the effects of antiestrogens administered at different stages in the menstrual cycle of non-human primates specially during the week following ovulation and mating. Catechol estrogens which represent metabolites of estrogens, are secreted by the blastocyst (rabbit) and may mediate in the stimulatory effects of estrogens on prostaglandin synthesis in the embryo and/or uterus and thus participate in the establishment of implantation. Catechol estrogens are formed uniquely in the embryo and it should therefore be possible to selectively interfere with the blastocyst by inhibitors/antagonists of catechol estrogens. However, such compounds need to be synthesized and evaluated.

#### *Luteolysis:*

The role of prostaglandins in implantation is not fully established. Earlier studies indicate a luteolytic role for prostaglandins (PGs) in a few species of animals other than non-human primates. The role of PGs in capillary permeability has been studied. If implantation is considered to involve inflammatory and immunosuppressant actions, perhaps PG-E<sub>2</sub> can do both, but in reality this class of PGs does not interfere with implantation. The cyclooxygenase system has been studied in relation to implantation recently. Leucotrienes have been identified in the blastocyst and these are known to have chemotactic action. Are these involved in the initiation of attachment of the blastocyst? This is an interesting area for further studies.

Monoclonal antibodies to LHRH agonist have been reported to terminate pregnancy in baboons when administered around the periimplantation period. It is suggested that this effect may be due to the direct effects on the trophoblast. Considerable work is needed to confirm this effect and establish the safety of a passive immunization approach for fertility regulation.

Luteinizing hormone receptor binding inhibitors (LHRBI) have been isolated from the follicular fluid and corpora lutea. The identity of these compounds, purification and demonstration of their effects *in vivo* need many years of basic research before they can be considered for use in regulation of fertility.

Deglycosylated derivatives of hCG have been shown to antagonize the action of hCG and interfere with established pregnancy in rats. Studies on the stability of the derivatives and their effects on the termination of pregnancy in non-human primates are in progress. Appropriate methods of delivery, possible immunogenicity and alternate methods of production of hCG need to be studied before evaluation of the derivatives for clinical use.

#### **Research needs**

There are a number of gaps in our knowledge of the processes regulating implantation which need to be investigated. Some of these are indicated in the preceding discussion. Other areas are:

- changes in the concentration of steroid receptors during the peri- and post-implantation period.

- are there any enzymes secreted by the blastocyst which are unique and crucial to the initiation of implantation?
- whether the functional life of the corpus luteum can be prolonged by hCG administration, if so, for how long? Such a model would be useful in screening luteolytic agents.
- There are at present no biochemical markers for the prediction of implantation. It is necessary to determine if some of the proteins secreted by the preimplantation blastocyst enter the saliva/urine and can be detected by simple tests. hCG has been detected in the preimplanting blastocyst but a diagnostic and sensitive test to determine its presence in saliva/urine as a method to predict implantation remains to be developed.
- develop more potent luteolytic agents. Deglycosylated hCG needs to be evaluated in non-human primates.
- Progesterone receptor blockers: recent reports on the development of a potent progesterone receptor blocker interfering with early pregnancy has evoked considerable interest in these compounds. There is need for the initiation of a programme of synthesis and evaluation of new classes of antiprogestins (steroidal or non-steroidal) based on an understanding of the structure of the progesterone receptor.

### Animal models

It is most unlikely that any of the rodent or non-human primate species will prove ideal as a model for all aspects of implantation in the human. It may be necessary to carry out some studies in the human after obtaining clearance from the appropriate Ethics Committees of the institutions where such studies are carried out and without compromising safety. Non-human primates are preferred for the reason that they are close to the human but their widespread use depends on a number of factors such as availability, cost, ease of management and captive breeding performance. It is necessary to establish international collaboration between countries/institutions where primate facilities exist to optimize their use in research on regulation of fertility. Likewise, there is need for standardization of methods and models and exchange of scientists and information to achieve the objectives outlined earlier.

### Status of research and development with post-coitals and menses inducers

Methods of post-coital contraception used so far include IUD's inserted post-coitally, progestogens used alone or in combination with estrogens. These are reserved primarily for emergency situations to protect women from unwanted pregnancy. Levonorgestrel, which has shown satisfactory contraceptive efficacy is being further evaluated clinically. Centchroman, a non-steroidal drug has been evaluated clinically as a once-a-week pill but its future as a fertility regulating agent depends on re-evaluation of its toxicology and determination of a suitable dose to avoid undesirable side effects.

Menstrual regulation could be achieved by a number of approaches: (a) block progesterone receptors and interfere with the preparation of the uterus for implan-

tation, (b) luteolysis leading to decreased progesterone levels and interruption of pregnancy and (c) termination of early pregnancy by prostaglandin analogues. A number of progesterone antagonists have been evaluated clinically. One of these compounds, RU-38486 has received considerable attention recently and is being evaluated clinically for termination of early pregnancy. Preliminary results seem to indicate that the drug, even in high doses, may not result in complete evacuation of the product of conception. A number of prostaglandin analogues are being evaluated clinically for termination of early pregnancy specially under conditions when they are self-administered at home.

It is perhaps reasonable to expect that a post-coital pill or a menstrual regulating agent based on ongoing clinical studies may be available for general use before the end of the decade. However, any method based on new leads which may emerge from basic research studies is unlikely to be available for clinical use in regulation of fertility before the turn of the century.